# RESEARCH ARTICLE

# Body weight-dependent troponin T alternative splicing is evolutionarily conserved from insects to mammals and is partially impaired in skeletal muscle of obese rats

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## SUMMARY

Do animals know at a physiological level how much they weigh, and, if so, do they make homeostatic adjustments in response to changes in body weight? Skeletal muscle is a likely tissue for such plasticity, as weight-bearing muscles receive mechanical feedback regarding body weight and consume ATP in order to generate forces sufficient to counteract gravity. Using rats, we examined how variation in body weight affected alternative splicing of fast skeletal muscle troponin T (*Tnnt3*), a component of the thin filament that regulates the actin–myosin interaction during contraction and modulates force output. In response to normal growth and experimental body weight increases, alternative splicing of *Tnnt3* in rat gastrocnemius muscle was adjusted in a quantitative fashion. The response depended on weight *per se*, as externally attached loads had the same effect as an equal change in actual body weight. Examining the association between *Tnnt3* alternative splicing and ATP consumption rate, we found that the *Tnnt3* splice form profile had a significant association with nocturnal energy expenditure, independently of effects of weight. For a subset of the *Tnnt3* splice forms, obese Zucker rats failed to make the same adjustments; that is, they did not show the same relationship between body weight and the relative abundance of five *Tnnt3* β splice forms (i.e. *Tnnt*3 β2–β5 and β8), four of which showed significant effects on nocturnal energy expenditure in Sprague–Dawley rats. Heavier obese Zucker rats displayed certain splice form relative abundances (e.g. *Tnnt3* β3) characteristic of much lighter, lean animals, resulting in a mismatch between body weight and muscle molecular composition. Consequently, we suggest that body weight-inappropriate skeletal muscle *Tnnt3* expression in obesity is a candidate mechanism for muscle weakness and reduced mobility. Weight-dependent quantitative variation in *Tnnt3* alternative splicing appears to be an evolutionarily conserved feature of skeletal muscle and provides a quantitative molecular marker to track how an animal perceives and responds to body weight.

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Key words: *Tnnt3*, body weight, weight sensing, alternative splicing, troponin T, energetics, metabolic rate, obesity, body composition, skeletal muscle, gastrocnemius, muscle performance.

## INTRODUCTION

Skeletal muscles must counteract gravity and adjust their size and/or performance to accommodate variation in body weight. Aside from hypertrophy, the molecular and biochemical mechanisms that muscles use to adjust to changes in body weight are poorly understood, but recent studies in mammals suggest a central role for regulation of sarcomere gene expression (Goldsmith et al., 2010; Kemp et al., 2009). Sarcomere genes encoding proteins that function at the interface between thin and thick filaments are of particular interest, as these play a large role in regulating muscle force output and energy consumption.

One such protein is skeletal muscle troponin T, a component of the troponin complex that regulates muscle contraction and force output (Brotto, 2005; Gomes et al., 2004; Ogut et al., 1999). Mammalian skeletal muscle troponin T is encoded by a slow (*Tnnt1*) and fast (*Tnnt3*) gene (Perry, 1998), the latter of which undergoes extensive alternative splicing. *Tnnt3* pre-mRNA comprises 18 exons, including a cassette of five alternatively spliced exons near the 5′ end and a mutually exclusive pair of exons near the 3′ end, allowing a possible 128 unique splice forms. Changes in the relative

abundance of different *Tnnt3* alternative splice forms is likely to be an important component of muscle plasticity, as it affects the calcium sensitivity of contraction in isolated striated muscle fibers (Briggs and Schachat, 1996; Brotto, 2005; Gomes et al., 2004; Ogut et al., 1999; Pan and Potter, 1992).

Alternative splicing of troponin T in mammals has been studied primarily in regard to qualitative shifts in protein isoform expression during early development, and in response to altered muscle use (Medford et al., 1984; Perry, 1998; Stefancsik et al., 2003; Stevens et al., 2003; Yu et al., 2006). A recent study of insect flight muscles demonstrated that there is precise quantitative variation in the relative abundance of alternatively spliced troponin T mRNA transcripts and protein isoforms in response to experimental manipulation of body weight and nutritional state (Marden et al., 2008). Here, we used rats to test the hypothesis that in a mammalian load-bearing (gastrocnemius) muscle, quantitative alternative pre-mRNA splicing of *Tnnt3* is similarly regulated in response to natural and experimentally imposed variation in body weight.

In addition to responding to body weight, the troponin T mRNA splice form profile in insect flight muscle is associated with

**A**



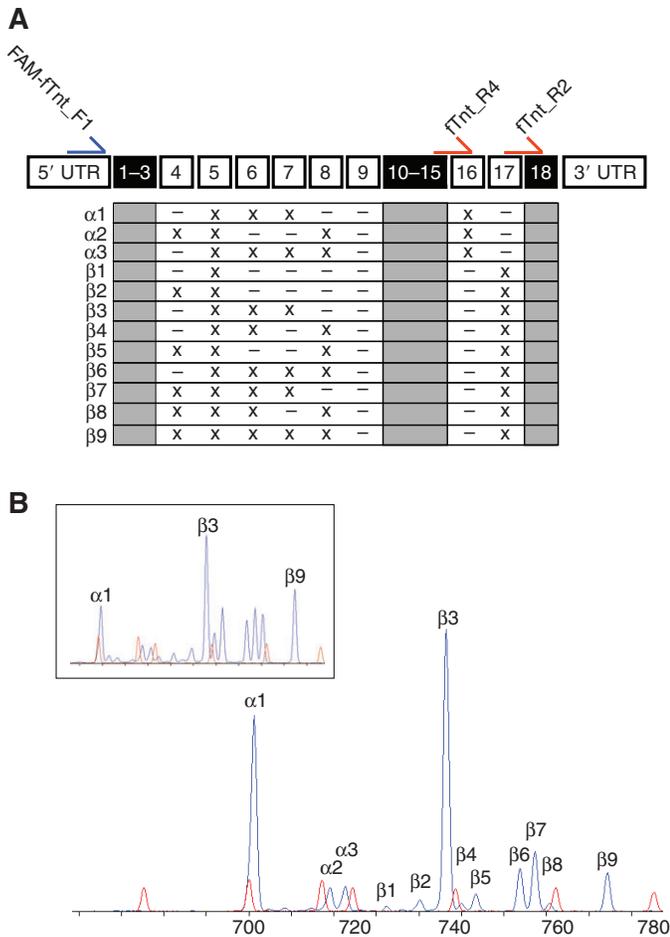| | 5′ UTR | 1–3 | 4 | 5 | 6 | 7 | 8 | 9 | 10–15 | 16 | 17 | 18 | 3′ UTR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| α1 | | | – | x | x | x | – | – | | x | – | | |
| α2 | | | x | x | – | – | x | – | | x | – | | |
| α3 | | | – | x | x | x | x | – | | x | – | | |
| β1 | | | – | x | – | – | – | – | | – | x | | |
| β2 | | | x | x | – | – | x | – | | – | x | | |
| β3 | | | – | x | x | x | – | – | | – | x | | |
| β4 | | | – | x | x | – | x | – | | – | x | | |
| β5 | | | x | x | – | – | x | – | | – | x | | |
| β6 | | | – | x | x | x | – | – | | – | x | | |
| β7 | | | x | x | x | x | – | – | | – | x | | |
| β8 | | | x | x | – | – | x | – | | – | x | | |
| β9 | | | x | x | x | x | x | – | | – | x | | |

**B**



Fig. 1. Characterization and quantification of *Tnnt3* alternative splicing in rat gastrocnemius muscle. (A) Rat *Tnnt3* pre-mRNA comprises 18 exons, including a 5′ alternatively spliced cassette containing exons 4–9 (white boxes), and the mutually exclusive exons 16 and 17. Twelve different *Tnnt3* splice forms were detected by RT-PCR and cDNA sequencing ('X' denotes inclusion of specific exons). Forward (FAM-fTnt_F1, blue) and reverse (fTnt_R2 and fTnt_R4, red) RT-PCR primers were designed so that each possible *Tnnt3* splice form amplicon size was unique and detectable by DNA fragment analysis (see Materials and methods). Splice forms containing exon 16 or exon 17 were given an 'α' or 'β' designation, respectively. (B) Fluorescently labeled DNA fragment peaks showing *Tnnt3* splice form diversity and abundance (i.e. peak height) of each splice form in gastrocnemius muscle of two rats differing in size (inset shows spectrum for the smaller of the two rats). Internal size standards are represented by red traces.

Nucleotide sequences were confirmed by cloning and sequencing each of the uniquely sized amplicons from PCR amplification of cDNA pooled from gastrocnemius muscles of a number of different-sized Sprague–Dawley rats. This amplicon pool was extracted from an agarose gel using a QiaQuick gel extraction kit (Qiagen, Valencia, CA, USA), cloned using a TOPO-TA cloning kit (Invitrogen) and sequenced (ABI Hitachi 3730XL DNA Analyzer, Applied Biosystems).

### *Tnnt3* protein (TNNT3) expression

Myofibrillar protein homogenates were prepared from flash-frozen gastrocnemius muscle samples as described previously (Hartner et al., 1989). The protein concentration of homogenates was determined (Bradford reagent, Bio-Rad, Hercules, CA, USA) and 15 μg of total protein per sample was loaded onto a 14% SDS-PAGE gel (acrylamide/bisacrylamide ratio 30:0.19). Electrophoretically separated proteins were transferred onto PVDF membranes, which were subsequently probed with a polyclonal anti-fast skeletal muscle troponin T antibody (C-18, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). TNNT3 protein bands were visualized using chemiluminescence (ECL Plus™, GE Healthcare, Chalfont St Giles, Bucks, UK) and a GeneGnome imaging system (Syngene, Frederick, MD, USA). Blots were stripped for 30 min at 55°C, in $0.063\,\mathrm{mol\,l^{-1}}$ Tris-Hcl, $0.07\,\mathrm{mol\,l^{-1}}$ sodium dodecyl sulfate (SDS) and $0.11\,\mathrm{mol\,l^{-1}}$ β-mercaptoethanol, pH 6.7, and reprobed with a monoclonal β-actin antibody (Sigma A5060, St Louis, MO, USA). Background-corrected, integrated optical band densities were quantified with Genetools (Syngene) software. Individual TNNT3 protein band abundance was expressed relative to the total abundance of all detected TNNT3 protein bands per sample. We also quantified β-actin protein abundance; this quantity was included in a statistical analysis to verify that covariance between relative abundance of TNNT3 isoforms and body mass occurred independently of variation in other sarcomere proteins and was not an artefact of differences in gel loading (see supplementary material Table S1).

### RESULTS
#### Alternative splicing of rat skeletal muscle *Tnnt3*

We detected 12 mature *Tnnt3* splice forms expressed in rat skeletal muscle (see Fig. 1A for the exon structure of each splice form) by means of RT-PCR screening of total RNA. Sequencing of cDNA clones showed that 9 of the 12 distinct mRNA splice forms contained exon 17 (henceforth referred to as *Tnnt3* β1–9), and three contained exon 16 (henceforth referred to as *Tnnt3* α1–3; Fig. 1A). These results are consistent with previous findings regarding the *Tnnt3* splice forms expressed in adult mammalian skeletal muscle (Briggs and Schachat, 1996), but extend considerably the known number of *Tnnt3* mRNA splice forms expressed in adult rodent muscle. To our knowledge, only seven adult *Tnnt3* mRNA splice forms have been reported previously (Wang and Jin, 1997).

#### Effects of weight manipulation on *Tnnt3* splicing

Weight loads caused pronounced effects on the *Tnnt3* mRNA splice form relative abundance, whereas wearing the unweighted vest had little if any effect (Table 1). The first principal component of variation in *Tnnt3* splice form relative abundance, characterizing the overall splice form mixture, was significantly related to body mass ($F$=101.2, $P<0.0001$) and weight load ($F$=22.3, $P<0.0001$). Individually, 8 out of 12 *Tnnt3* mRNA splice forms responded to total mass, with body mass and weight load showing highly significant additive effects (Table 1). For example, the *Tnnt3* α1 relative abundance in weight-loaded rats was indistinguishable from that in unloaded rats of the same total mass (Fig. 2A). Not all splice forms responded to weight in the same fashion (Fig. 2B), as certain ones (e.g. *Tnnt3* β2; Fig. 2B) were completely non-responsive to weight. *Tnnt3* β3 showed a mixed response, increasing in relative abundance in rats weighing ~100–250 g, but with no further increase at higher body mass.

Weight-dependent changes in *Tnnt3* expression were also evident at the protein level, albeit less well resolved given the complexity of the isoform mixture. The relationship between body mass and the relative abundance of the two most abundant TNNT3 bands (bands 2 and 4 in Fig. 3A) followed the same pattern as the two most abundant mRNA splice forms (i.e. *Tnnt3* β3 and *Tnnt3* α1; compare Fig 2 and Fig. 3B). Thus, although we did not fully characterize these protein bands, there were clear body weight-associated shifts in relative TNNT3 band abundance that correlated with the more readily measured changes at the mRNA level.

Table 1. *F*-statistics and associated *P*-values for effects of body mass, weight load and presence of a vest on *Tnnt3* splice form relative abundance

| *Tnnt3* splice form | Model effects | | | | | |
|---|---|---|---|---|---|---|
| | Body mass | | Weight load | | Vest (yes/no) | |
| α1 | 90.5 | (<0.0001) | 27.30 | (<0.0001) | 2.20 | (0.15) |
| α2 | 27.1 | (<0.0001) | 7.10 | (0.01) | 1.40 | (0.25) |
| α3 | 13.2 | (0.0007) | 9.20 | (0.004) | 2.10 | (0.16) |
| β1 | 41.2 | (<0.0001) | 6.20 | (0.016) | 1.40 | (0.25) |
| β2 | 1.5 | (0.23) | 0.67 | (0.42) | 0.31 | (0.58) |
| β3 | 32.4 | (<0.0001) | 0.06 | (0.81) | 3.03 | (0.09) |
| β4 | 0.07 | (0.80) | 5.48 | (0.024) | 5.25 | (0.03) |
| β5 | 62.9 | (<0.0001) | 3.86 | (0.055) | 0.05 | (0.82) |
| β6 | 14.3 | (0.0004) | 15.30 | (0.0003) | 0.38 | (0.54) |
| β7 | 21.0 | (<0.0001) | 3.18 | (0.08) | 0.04 | (0.85) |
| β8 | 22.7 | (<0.0001) | 6.24 | (0.016) | 7.83 | (0.0075) |
| β9 | 104.2 | (<0.0001) | 10.95 | (0.0018) | 3.40 | (0.07) |
| PC1 | 101.2 | (<0.0001) | 22.3 | (<0.0001) | 3.9 | (0.06) |

Results of ANCOVA models (*N*=50, α=0.05) predicting the effects of body mass, added weight load and presence of a vest on relative *Tnnt3* splice form abundance (d.f.=1 for each predictor) in male Sprague–Dawley rats. *P*-values are given in parentheses.
Significant *P*-values for body mass indicate that *Tnnt3* splice form abundance responds to natural variation in body mass.
Significant *P*-values for weight load indicate that, independently of natural body mass variation, *Tnnt3* splice form abundance responds to experimental manipulation of body weight.
The bottom row shows that both body mass and added weight load are highly significant predictors of the first principal component (PC1) of the *Tnnt3* splice form relative abundance.

### *Tnnt3* splice form abundance is associated with energy expenditure during activity

Changes in *Tnnt3* splice form abundance in response to weight loading were not associated with gross alterations in activity or energy expenditure (Fig. 4), as weight-loaded and control rats showed no differences in ambulatory activity, energy expenditure ($\dot{V}_{O_2}$) or RER (i.e. $\dot{V}_{CO_2}/\dot{V}_{O_2}$). Moreover, a multiple regression model showed that ambulatory activity was not significantly associated
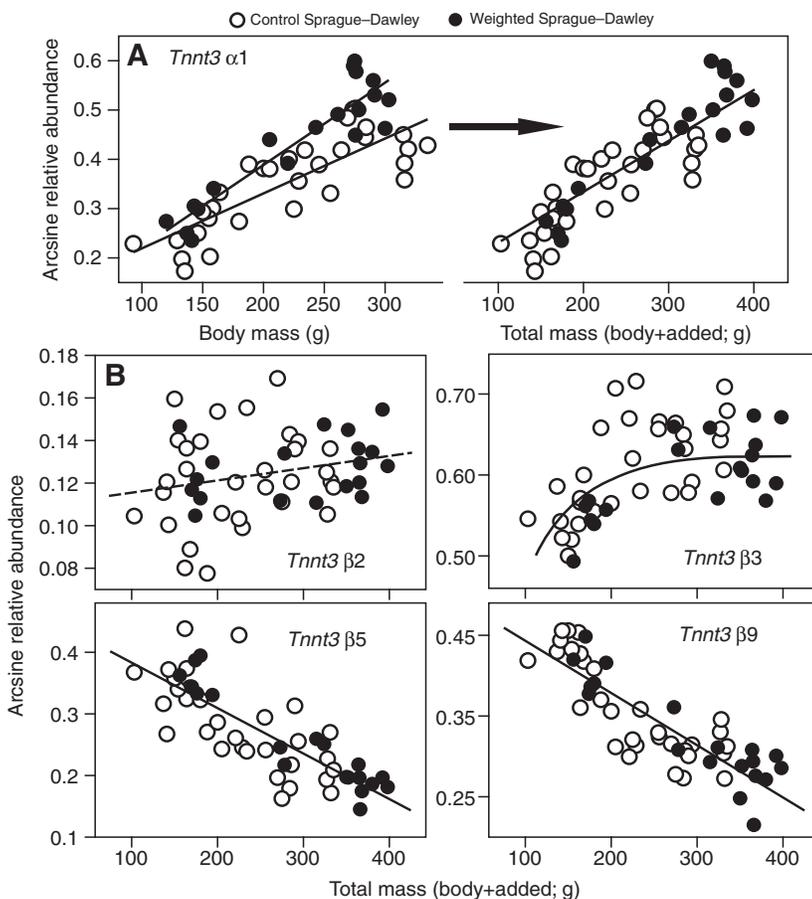


Fig. 2. *Tnnt3* splice form abundance is adjusted to total mass in rat gastrocnemius muscle. (A) Relative abundance of *Tnnt3* splice form α1 as a function of native body mass (left) and total mass (body+added load; right) for weight-loaded and control male Sprague–Dawley rats. Solid lines depict linear regression fits to data for weight-loaded (*R*²=0.84, *P*<0.0001, *N*=19) and control animals (*R*²=0.67, *P*<0.0001, *N*=31), separately (left panel) or combined (right panel) (*R*²=0.78, *P*<0.0001). (B) Expression of *Tnnt3* splice form β2 (plotted here against total load) was not sensitive to native or experimental changes in body weight. *Tnnt3* splice form β3 expression initially increased with total mass, but leveled off in rats >250 g. Both *Tnnt3* β5 and β9 expression correlated negatively with total mass and were sensitive (*Tnnt3* β5 marginally so) to experimental body weight manipulation (see Table 1). All relative abundance data were arcsine transformed to achieve normality.
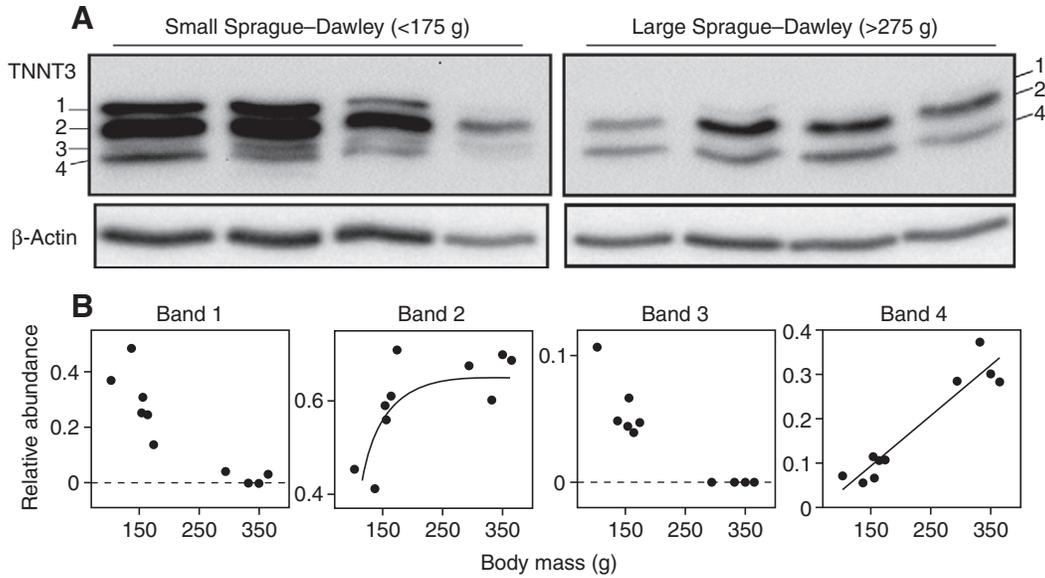
Fig. 3. Weight-associated changes in TNNT3 protein bands. (A) A western blot for TNNT3 detected four bands in smaller Sprague–Dawley rats, whereas three bands (i.e. bands 1, 2 and 4) were apparent in larger rats. β-Actin levels (obtained after stripping and reprobing the same western blot) are provided to demonstrate relatively uniform protein loading. β-Actin levels were not significantly affected by changes in body weight (one-way ANOVA: $F$=2.57, $P$=0.163). (B) Relative abundance of TNNT3 protein bands in relation to body mass. In a sample of gastrocnemius muscles from 10 Sprague–Dawley individuals, the most abundant TNNT3 protein (band 2) matched the behavior of the most abundant mRNA splice form (*Tnnt3* β3; Fig. 2B) in regard to its relationship with body mass (i.e. increasing at low body mass and leveling off above 250 g). The second most abundant TNNT3 protein, band 4, and the second most abundant mRNA splice form, *Tnnt3* α1 (Fig. 2A), in large rats showed the same linear increase with body mass. These trends associated with body mass increase were evident when variation associated with protein loading was taken into account as a three-way ANOVA (i.e. with TNNT3 band relative abundance as responses, and total TNNT3 band density, β-actin density and body mass as factors) revealed significant effects of body mass only (supplementary material Table S1).

with either rat size (body mass; $F$=2.21, $P$=0.16) or the amount of weight load ($F$=0.11, $P$=0.75).

To more closely examine the effect of the *Tnnt3* splice form profile on energy expenditure, we controlled for individual differences that would otherwise inflate the error variance and reduce statistical power. In this analysis, the response variable was night-

time $\dot{V}_{O_2}$, the period when rats were most active. We included as independent variables the day-time $\dot{V}_{O_2}$, to account for individual variation in size and resting metabolic rate, and night-time ambulatory activity to control for activity level (Table 2). The relative abundance of *Tnnt3* splice forms had significant independent effects on night-time $\dot{V}_{O_2}$ independently of weight load (Table 2). Rats with
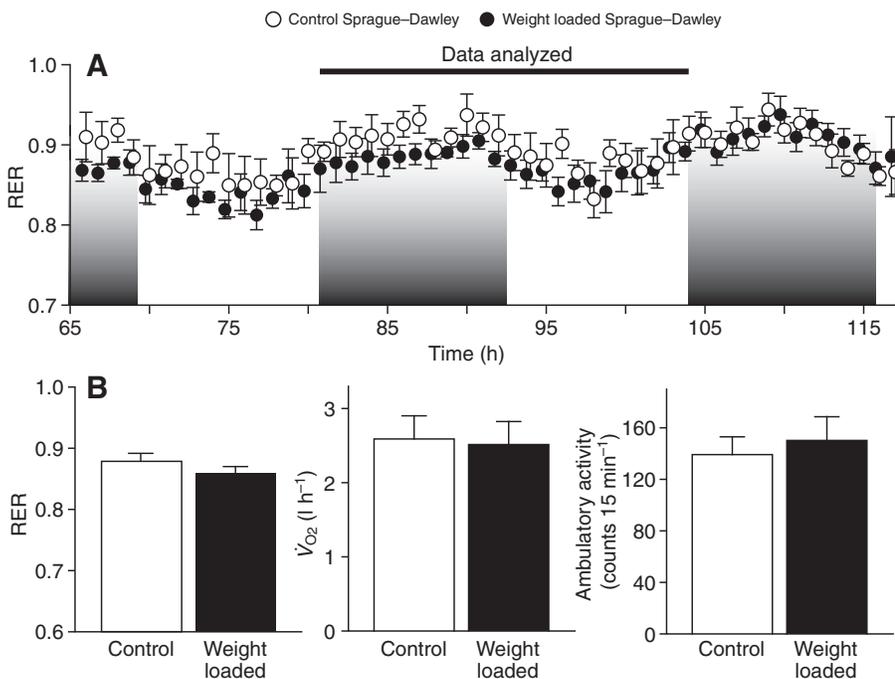


Fig. 4. Experimental manipulation of body weight did not affect metabolism, locomotor activity or energy expenditure. (A) Mean respiratory exchange ratio (RER, i.e. $\dot{V}_{CO_2}/\dot{V}_{O_2}$) as a function of time for weight-loaded and control rats maintained in indirect calorimetry chambers for 5 days, during the last ~50 h of the experiment ($N$=16 rats). Shaded areas indicate the dark period of the 12 h light/dark cycle. Statistical analyses of the effects of weight loading on energy expenditure and activity (see also Table 3) were performed on data obtained during the last full day (i.e. animals had been weight loaded for 4 days). (B) Comparison of mean RER (0.88±0.009 *vs* 0.86±0.01), mean $\dot{V}_{O_2}$ (2589.3±312.2 *vs* 2514.2±310.2 ml h$^{-1}$) and mean ambulatory activity (139.2±13.8 *vs* 150.2±18.5 counts 15 min$^{-1}$) during day 4. No differences were detected between weight-loaded and control animals. Means are presented with s.e.m. (error bars).

Table 2. *F*-statistics and associated *P*-values for effects of night-time ambulatory activity and weight load, and the relative abundance of each of 12 *Tnnt3* splice forms on mean night-time $\dot{V}_{O_2}$

| *Tnnt3* splice form | Model effects | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Relative abundance | | | NAA | | Weight load | | |
| α1 | 10.50 | (0.008) | + | 0.5 | (0.5) | 5.6 | (0.04) | |
| α2 | 4.71 | (0.053) | | 0.030 | (0.9) | 1.9 | (0.2) | |
| α3 | 0.91 | (0.36) | | 0.003 | (0.96) | 0.6 | (0.4) | |
| β1 | 10.61 | (0.008) | – | 1.82 | (0.20) | 5.1 | (0.045) | |
| β2 | 0.02 | (0.90) | | 0.002 | (0.97) | 0.02 | (0.9) | |
| β3 | 9.22 | (0.01) | + | 0.007 | (0.93) | 0.34 | (0.5) | |
| β4 | 6.21 | (0.03) | – | 0.1 | (0.8) | 3.5 | (0.09) | |
| β5 | 4.04 | (0.07) | | 0.005 | (0.94) | 0.98 | (0.34) | |
| β6 | 0.47 | (0.51) | | 0.09 | (0.8) | 0.4 | (0.5) | |
| β7 | 7.43 | (0.02) | – | 0.01 | (0.92) | 2.1 | (0.2) | |
| β8 | 8.76 | (0.013) | – | 0.000 | (0.995) | 2.9 | (0.12) | |
| β9 | 10.89 | (0.007) | – | 0.002 | (0.97) | 4.1 | (0.07) | |
| PC1 | 9.80 | (0.0097) | | 0.14 | (0.72) | 4.8 | (0.05) | |

Results of multiple regression models ($N$=16, $\alpha$=0.05; *P*-values are given in parentheses) for energy expenditure during activity (i.e. mean night-time $\dot{V}_{O_2}$), using weight load and each *Tnnt3* splice form as predictors (d.f.=1, for all predictors). Also included in each of these models were night-time ambulatory activity (NAA) and mean daytime $\dot{V}_{O_2}$. *P*-values for the latter were omitted from this table ($P$<0.0001 for all nine models), as this factor was primarily included to account for size-dependent variation in resting metabolic rate.

Significant *P*-values for a *Tnnt3* splice form indicate that its relative abundance affects energy expenditure during activity, independently of weight, NAA and mean daytime $\dot{V}_{O_2}$.

The + and – designations in the *Tnnt3* splice form relative abundance column represent the directionality of the splice form-specific effects on mean night-time $\dot{V}_{O_2}$.

The bottom row shows that both the first principal component (PC1) of the *Tnnt3* splice form relative abundance and weight load had independent, significant effects on energy expenditure during activity.

muscles containing more *Tnnt3* α1 and β3 at a given daytime $\dot{V}_{O_2}$ and weight load expended energy at a higher rate (see directionality of effects in Table 2), as expected if the functional effect of expressing proportionately more of these splice forms increases force and power. *Tnnt3* α1 and β3 are among the smaller *Tnnt3* splice forms and have identical 5′ exon composition (Fig. 1A), and hence these energetic results are consistent with a previous qualitative finding that unloaded soleus muscles [hindlimb-suspended rats (Yu et al., 2006)] had reduced expression of smaller *Tnnt3* isoforms and produced less force (i.e. opposite to the effects we observed in response to weight loading).

### Regulation of *Tnnt3* alternative splicing is impaired in obese rats

Fat loads of obese Zucker rats in relation to lean mass were similar to the exogenously applied loads in the foregoing experiment (ANCOVA *post hoc* Student's *t*-test: $\alpha$=0.05, $t$=0.96, $P$=0.34; Fig. 5B) and hence the skeletal muscles were loaded in a comparable

Table 3. *F*-statistics and associated *P*-values for effects of body mass, Zucker rat strain and interaction term on *Tnnt3* splice form relative abundance

| *Tnnt3* splice form | Model effects | | | | | |
|---|---|---|---|---|---|---|
| | Body mass | | Obese/lean | | Body mass × obese/lean | |
| α1 | 90.5 | (<0.0001) | 27.30 | (<0.0001) | 2.20 | (0.15) |
| α1 | 11.7 | (0.003) | 0.45 | (0.5) | 0 | (0.998) |
| α2 | 0.32 | (0.58) | 0.45 | (0.5) | 0.001 | (0.97) |
| α3 | 0.02 | (0.9) | 0.5 | (0.5) | 0.11 | (0.74) |
| β1 | 21.1 | (0.0002) | 0.1 | (0.8) | 8.1 | (0.01) |
| β2 | 6.0 | (0.02) | 3.9 | (0.06) | 0.07 | (0.80) |
| β3 | 36.8 | (<0.0001) | 80.6 | (<0.0001) | 9.1 | (0.007) |
| β4 | 1.4 | (0.3) | 12.4 | (0.002) | 5.2 | (0.03) |
| β5 | 13.4 | (0.002) | 3.8 | (0.007) | 9.8 | (0.005) |
| β6 | 36.6 | (<0.0001) | 0.32 | (0.58) | 0.97 | (0.34) |
| β7 | 7.99 | (0.01) | 1.27 | (0.27) | 0.02 | (0.90) |
| β8 | 1.63 | (0.22) | 13.20 | (0.0017) | 0.59 | (0.45) |
| β9 | 14.8 | (0.001) | 2.80 | (0.11) | 0.45 | (0.51) |
| PC1 | 19.8 | (0.0003) | 5.6 | (0.03) | 0.2 | (0.7) |

Results of ANCOVA models ($N$=22, $\alpha$=0.05; *P*-values are given in parentheses) predicting the effects of body mass and Zucker rat strain (obese/lean) on the relative abundance of *Tnnt3* splice forms. Inclusion of the interaction term allowed us to examine whether *Tnnt3* splicing responded to body mass change as the severity of obesity progressed.

Of particular interest are *Tnnt3* splice forms β3 and β5, which showed significant responses to body mass, and highly significant interaction terms indicating obesity effects that were body mass dependent (i.e. depending on the severity of obesity; see Fig. 5B for visual representation of these effects).

The bottom row shows that body mass and obesity had significant effects on PC1 for Zucker rats.
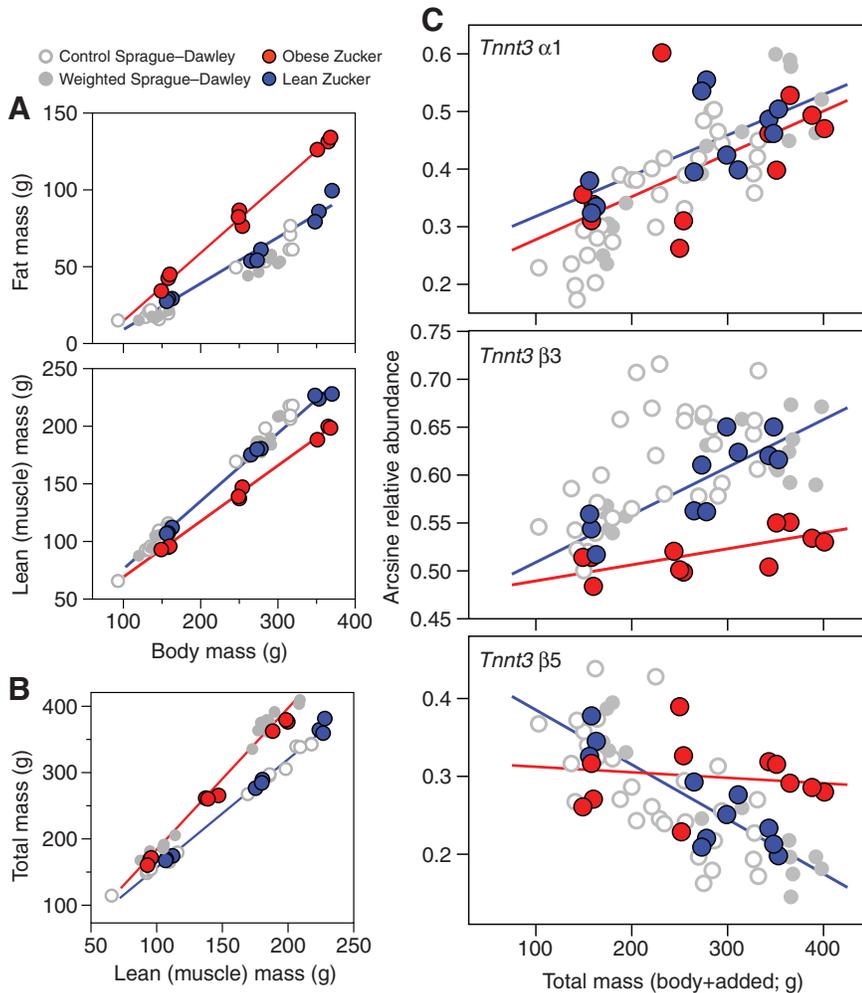
Fig. 5. Body composition and *Tnnt3* splicing response to changes in body weight in lean and obese Zucker rats. Body composition and *Tnnt3* relative abundance data for Zucker rats are superimposed on data for Sprague–Dawley rats. (A) Body fat and lean mass as a function of body mass in male lean and obese Zucker rats (*N*=18). (B) Lean mass contribution to the total weight experienced by skeletal muscles was similar in obese Zucker and weight-loaded Sprague–Dawley rats, indicating that muscles were loaded equally in these groups. (C) Comparison of relative abundance for 3 out of 12 *Tnnt3* splice forms between obese and lean Zucker rats across a range of total mass (*N*=22; see Table 3 for statistical analyses of all *Tnnt3* splice forms). The splicing response to changes in total mass for *Tnnt3* α1 is unaffected by obesity. Impaired responses were observed for *Tnnt3* β3 and β5 (and β1, β8 and the first principal component characterizing the overall mixture; see Table 3). The Sprague–Dawley data are shown here only for reference and were excluded from all linear regression fits.

fashion across the two experiments (body composition data are presented in Fig. 5A,B, and statistical comparisons in supplementary material Table S2).

*Tnnt3* expression in lean Zucker rats showed the same relationship to total mass as we observed in Sprague–Dawley rats (Fig. 5C). In contrast, obese Zucker rats showed an impaired body weight-dependent response in the β-splice forms (Fig. 5C and Table 3), i.e. those that contain the 3′ exon 17, whereas the α-splice forms behaved in the normal fashion. For some of the *Tnnt3* β-splice forms, including the most abundant transcript, *Tnnt3* β3, obese rats had a splice form relative abundance normally observed in rats of much lower body mass (e.g. 350 g obese rats had a *Tnnt3* β3 relative abundance matching that of 150 g lean rats; Fig. 5C). The mismatch between *Tnnt3* splice form profile and body mass in obese rats increased as body mass increased (e.g. *Tnnt3* β3; Fig. 5C and significant interaction effects in Table 3).

The mismatch between *Tnnt3* mRNA splice form profile and body mass in obese rats was accompanied by deviations from the normal troponin T pattern at the protein level (Fig. 6A,B). Large obese Zucker rats showed TNNT3 band patterns that qualitatively resembled those of much smaller Sprague–Dawley and lean Zucker rats (Fig. 6A). Large obese Zucker rats showed an altered relative abundance of TNNT3 bands 2 and 3 compared with large Sprague–Dawley (see also Fig. 3B) and lean Zucker rats (Fig. 6B). For TNNT3 band 1, the pattern shown in Fig. 3B for Sprague–Dawley rats (i.e. decrease with increasing body mass) was

not replicated in a second set of Sprague–Dawley and Zucker rats (Fig. 6). The high variability of this band across the two western blots (Fig. 3B, Fig. 6B) may reflect expression of a mRNA splice form(s) that is not consistently related to body mass (e.g. as we saw at the mRNA level for *Tnnt3* β2 or *Tnnt3* β4, Table 1).

## DISCUSSION

Our findings show that quantitative variation in rat skeletal muscle *Tnnt3* splice form abundance is regulated in response to natural and experimental variation in body weight. This response depends on weight *per se*, rather than on other hidden correlates of body mass, as externally attached weight loads had the same effect as an equal change in body mass due to growth (Fig. 2A). Given the known effect of *Tnnt3* protein isoforms on muscle force and sensitivity to activation by calcium (Brotto, 2005; Gomes et al., 2004; Ogut et al., 1999), the function of this molecular change may be to adjust skeletal muscle mechanical performance in response to load, with consequent effects on force output and energy expenditure during activity (e.g. Table 2). Indeed, the overall *Tnnt3* splicing adjustments associated with body weight gain observed in this study consisted of increases in the relative abundance of *Tnnt3* splice forms that exclude exon 4 (e.g. *Tnnt3* α1 and β3), and decreases in those that include exon 4 (e.g. *Tnnt3* β5 and β9). Reduced inclusion of exon 4 is known to correlate with increased muscle fiber calcium sensitivity (Briggs and Schachat, 1996; Schachat et al., 1987). Hence, we predict that the overall effect of natural and experimental
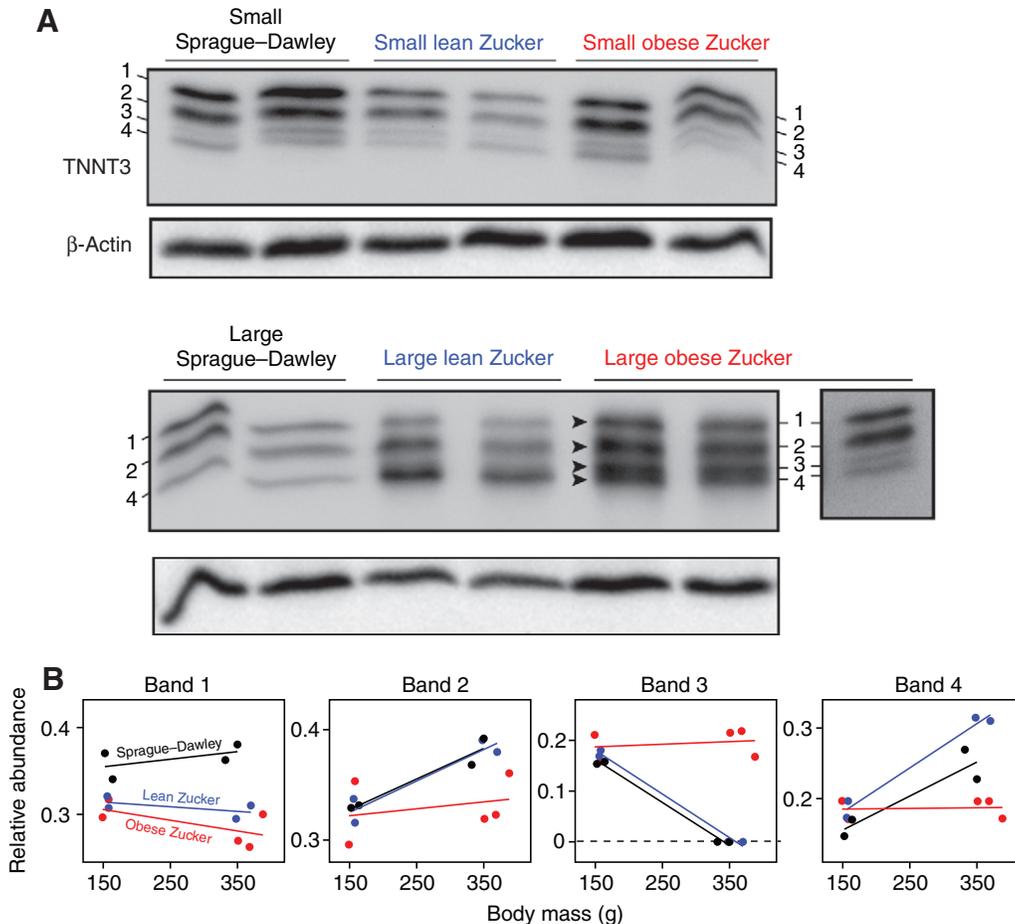
Fig. 6. Weight-associated changes in TNNT3 protein band abundance in Sprague–Dawley and Zucker rat gastrocnemius muscle. (A) A western blot for TNNT3 detected four bands in small rats (i.e. 149–164 g), whereas the large Sprague–Dawley and lean Zucker rats (i.e. 332–370 g) displayed three bands. In large obese Zucker rats (i.e. 351–388 g), an expression pattern similar to that of small rats (i.e. four bands, indicated by black arrowheads) was observed. Upper and lower main panels originate from the same western blot, but are presented separately. β-Actin levels (obtained after stripping and reprobing the same western blot) are provided to demonstrate relatively uniform protein loading. β-Actin levels were not significantly affected by changes in body weight (one-way ANOVA: $F=1.54$, $P=0.243$). The small panel on the lower right of part A originates from a separate western blot and differs in exposure from the two main panels. This panel provides a more readily perceivable visualization of the small rat-like band pattern in large obese Zucker rats, but these band intensities were not used for quantification. (B) Relative abundance of TNNT3 protein bands in relation to body mass for Sprague–Dawley rats and lean and obese Zucker rats. Data fits are provided to demonstrate trends only, as sample sizes are insufficient for statistical inference. Large obese Zucker rat TNNT3 band relative abundance differed from that of Sprague–Dawley and lean Zucker rats for bands 2 and 3 in particular.

increases in body weight is an increased calcium sensitivity and force output in gastrocnemius and perhaps other weight-bearing muscles. We are currently testing these predictions.

In obese Zucker rats, this response was impaired, causing a mismatch between body mass and certain components of skeletal muscle *Tnnt3* composition that became more pronounced as obesity increased (Fig. 5C, Table 3). This mismatch includes *Tnnt3* β3, β4, β5, β6 and β8 (Fig. 5C); transcripts that respond to native and/or applied weight loads (except *Tnnt3* β4, see Table 1), and are associated with nocturnal energy expenditure rate (except *Tnnt3* β6, see Table 2). For *Tnnt3* β3–β5, there were significant interaction effects between body mass and obesity status, indicating that the weight dependency of the relative abundance of these *Tnnt3* splice forms changed as body size increased and obesity progressed. Thus, obese Zucker rats appear to partially, yet progressively, lose the ability to homeostatically regulate their skeletal muscle molecular composition, starting at an early point in the development of obesity.

Moreover, from the previous paragraph it follows that the dramatic reduction of *Tnnt3* β3 and increase in *Tnnt3* β5 mRNA relative abundance (i.e. relatively more exon 4 inclusion in the overall *Tnnt3* splice form pool) in larger obese Zucker rats would lead to decreased calcium sensitivity and hence force output by their gastrocnemius muscles.

The resulting mismatch between body weight and skeletal muscle molecular composition (and presumably performance) may be a component of pathologies common in human obesity, such as chronic muscle weakness, increased load-induced muscle injury and unwillingness to participate in exercise (Anandacoomarasamy et al., 2008; Levine et al., 2005).

Quantitative changes in the *Tnnt3* isoform profile at the mRNA level were paralleled by qualitative changes at the protein level, in both Sprague–Dawley and Zucker rats, including obese Zucker rats. Achieving a more precise understanding of the relationship between transcript and protein expression of this gene is extremely problematic, as the *Tnnt3* α- and β-splice forms differ at the mutually exclusive exon pair near the 3′ end, and each exists with varying combinations of alternative exons near the 5′ end of the gene. Hence, antibodies specific for α and β proteins would hybridize with a

mixture of different TNNT3 isoforms. For this reason, we have restricted our protein-level analysis of this gene to qualitative and simple quantitative examination.

Zucker rats become obese at an early age because of a genetic impairment in leptin signaling (Bray, 1977), and therefore it remains to be determined whether the effects observed in this study are specific to this model system or are associated generally with the development of obesity *per se*. Investigating the *Tnnt3* splicing response to the development of obesity caused by a high fat diet in otherwise healthy rats will help to clarify this. Nutritional regulation of pre-mRNA alternative splicing has not been examined in great detail (but see Salati and Amir-Ahmady, 2001; Salati et al., 2004) but, interestingly, in addition to body weight, alternative splicing of insect muscle troponin T is sensitive to nutrition (Marden et al., 2008). Moreover, alternative splicing of the vertebrate insulin receptor gene, a key component of nutrient signaling, is co-regulated with that of troponin T (Ho et al., 2004). Hence, it is possible that signals involving energy homeostasis commonly affect the regulation of troponin T splicing.

Research on mechanisms controlling body weight homeostasis has focused primarily on neuroendocrine regulation of food intake, satiety and energy storage depot size (Levin, 2006; McMinn et al., 2000; Morton et al., 2006; Schwartz et al., 2000). In concert with such mechanisms, our results suggest that there may be an important role for the skeletal musculature in maintaining proper body weight homeostasis. Healthy skeletal muscles apparently receive signals (intracellular and/or extracellular) about the amount of load they experience, and respond by changing their *Tnnt3* isoform composition. Elucidating the signaling pathways responsible for this regulatory response may improve our understanding of muscle function and identify targets for therapeutic intervention to reduce muscle pathologies in obesity.

Molecular signaling pathways mediating mechanical load-associated effects on muscle physiology are poorly understood, and have been examined primarily in terms of their involvement in skeletal muscle protein synthesis and growth (e.g. Atherton et al., 2009; Hornberger et al., 2005; Spangenburg, 2009). It is becoming apparent that many alternative splicing events require the activity of the same signaling pathways (Lynch, 2007). For example, the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR (mammalian target of rapamycin) signaling cascade mediates skeletal muscle protein synthesis in response to nutritional and mechanical stimuli (Anthony et al., 2000; Bolster et al., 2003; Kimball and Jefferson, 2006; Kimball et al., 2000; Sasai et al., 2010), and also activates and interacts with members of the serine/arginine (SR)-rich protein family (Blaustein et al., 2009; Karni et al., 2008; Lynch, 2007), a ubiquitous set of alternative splicing factors that affect exon inclusion/exclusion and facilitate translation of specific mRNAs (Blaustein et al., 2005; Sanford et al., 2005). Modulation of sarcomere gene alternative splicing by nutritional and/or mechanically induced signaling through PI3K/Akt/mTOR would add important functionality to the already complex nature of this pathway, and may mediate the dietary and weight-loading effects on troponin T alternative splicing observed in both insects and mammals.

Impairment of the normal *Tnnt3* splicing response in obese rats is restricted to the β-splice forms (see Fig. 5C and Table 3, i.e. those that contain the 3′ exon 17) (Pan and Potter, 1992). This result implies that a particular aspect of the regulation of alternative splicing (i.e. 3′ end exon inclusion) is modified during obesity. This specificity may help to guide future examination of the signaling pathways responsible. Obesity is associated with a chronic inflammatory state mediated by the infiltration of adipose tissue by macrophages (Wisse, 2004).
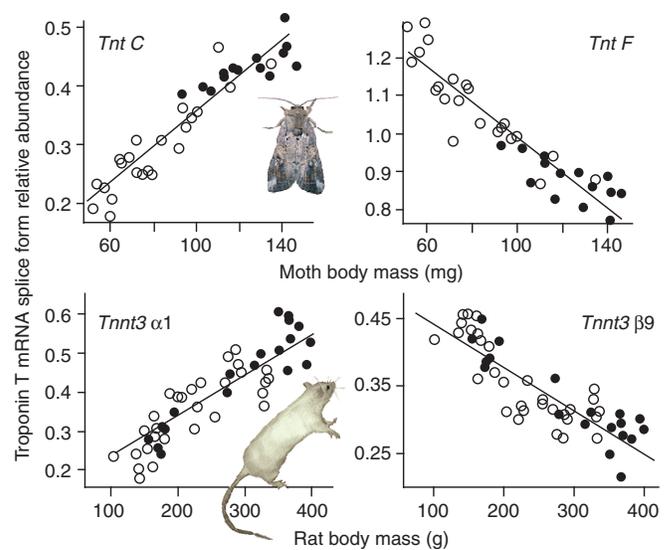


Fig. 7. Comparison of body weight-dependent *troponin T* alternative splicing in insects and mammals. Insects [upper panels; graphs are modified from data originally published in Marden et al. (Marden et al., 2008)] and mammals (lower panels; data from this study) show very similar body weight-dependent reaction norms for the troponin T mRNA splice form profile. In both taxa the response is identical for growth-related changes in body mass (open circles) and experimental weight loading (filled circles). There is not a direct homology of the exon structure of the alternative troponin T mRNA transcripts for which relative abundance is shown here; precisely how these molecular variations affect function in either taxa remains to be determined.

Importantly, inflammation in skeletal muscle impairs splice factor expression (Xiong et al., 2006), and attenuates Akt (Varma et al., 2009) and mTOR signaling (Lang et al., 2007). Indeed, skeletal muscle in rodent models of obesity (including Zucker rats) shows impaired Akt and mTOR signaling (Katta et al., 2009; Kim et al., 2000), which reinforces that idea that these pathways are prime candidates to examine in regard to the impairment in *Tnnt3* splicing we observed.

More broadly, these results indicate that skeletal muscle composition is regulated to accommodate performance requirements associated with changing body weight in a fashion that is evolutionarily conserved in animals ranging from insects (Marden et al., 2008) to mammals (illustrated in Fig. 7). Such a response may be necessary because muscle force scales with cross-sectional area whereas gravitational load scales with body mass. For dimensionally similar animals (i.e. proportions of linear dimensions are invariant), increasing muscle mass alone will not maintain a constant ratio of force to body weight as animals grow larger (Biewener, 1989). The ability to modulate performance by changing muscle *Tnnt3* isoform composition may at least partially circumvent such geometric constraints.

In summary, our findings provide a new mammalian model system to study the quantitative regulation of alternative splicing, and a quantitative marker for an evolutionarily conserved homeostatic mechanism by which animals respond to body weight. Identifying the body weight-sensitive pathways that regulate *Tnnt3* alternative splicing, and the dysregulation thereof during obesity, may reveal new ways to approach the biomedicine of body weight regulation in health and disease.

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