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RESEARCH ARTICLE

Molecular plasticity and functional enhancements of leg muscles in response to hypergravity in the fruit fly *Drosophila melanogaster*

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ABSTRACT

Studies of organismal and tissue biomechanics have clearly demonstrated that musculoskeletal design is strongly dependent on experienced loads, which can vary in the short term, as a result of growth during life history and during the evolution of animal body size. However, how animals actually perceive and make adjustments to their load-bearing musculoskeletal elements that accommodate variation in their body weight is poorly understood. We developed an experimental model system that can be used to start addressing these open questions, and uses hypergravity centrifugation to experimentally manipulate the loads experienced by *Drosophila melanogaster*. We examined effects of this manipulation on leg muscle alternative splicing of the sarcomere gene troponin T (*Dmel \up*; Fbgn0004169, herein referred to by its synonym *TnT*), a process that was previously demonstrated to precisely correlate with quantitative variation in body weight in Lepidoptera and rat. In a similar fashion, hypergravity centrifugation caused fast (i.e. within 24 h) changes to fly leg muscle *TnT* alternative splicing that correlated with body weight variation across eight *D. melanogaster* lines. Hypergravity treatment also appeared to enhance leg muscle function, as centrifuged flies showed an increased negative geotaxis response and jump ability. Although the identity and location of the sensors and effectors involved remains unknown, our results provide further support for the existence of an evolutionarily conserved mechanism that translates signals that encode body weight into appropriate skeletal muscle molecular and functional responses.

KEY WORDS: Musculoskeletal, Body mass, Centrifugation, Troponin T, Body weight, Alternative splicing, Weight sensing, Muscle performance

INTRODUCTION

A key requirement for animal locomotion is a musculoskeletal system that supports the weight of the body as it moves. For most species, body weight is not a static feature but can vary significantly across life history stages and among individuals, yet locomotion is achieved by all. Successful body weight support implies that there is feedback between sensors producing quantitative signals that somehow encode body weight and determinants of musculoskeletal function and plasticity (Schilder, 2016). In other words, animals should be able to perceive how much they weigh and appropriately adjust the design and performance of their musculoskeletal systems. This concept is fundamental to many comparative studies of body mass-dependent scaling of gross-level animal musculoskeletal design (e.g. Alexander, 1976, 2003; Alexander and Jayes, 1983; Biewener, 1989, 2005), whole-organismal performance limits (e.g. Marden, 1987, 2005; Marden and Allen, 2002) and skeletal muscle functional properties (Alexander, 1985; James et al., 1998; Medler, 2002; Schiaffino and Reggiani, 2011; Van Wassenbergh et al., 2007). It has also provided a conceptual framework for experimental studies of mechanical loading on muscle tissue growth and protein synthesis (Atherton et al., 2009; Burkholder, 2007; Goldspink, 1999; Hornberger et al., 2005; Spangenburg, 2009), and specific muscle sarcomere protein expression (e.g. Bastide et al., 2002; Bey et al., 2003; Pandorf et al., 2009; Yu et al., 2006). These studies have demonstrated that molecular features of musculoskeletal systems are sensitive to externally applied loads, although the described effects tend to be qualitative in nature. Thus, while several major research fields clearly suggest that there are evolutionarily conserved mechanisms that can accurately tune musculoskeletal designs to quantitative variation in body weight at both morphological and molecular levels, this has not yet been explicitly demonstrated.

Adjusting the molecular level design of animal musculoskeletal systems in a quantitatively precise fashion to body weight variation allows changes to their properties and function in ways that can circumvent the need for bulk protein synthesis and tissue mass (morphological) change, e.g. through differential gene and protein expression. For insect skeletal muscle, the subject of this study, it may be the dominant mechanism to achieve such plasticity, as significant muscle mass enhancement is often prevented by their rigid exoskeleton. As a case in point, flight muscles of the moth *Spodoptera frugiperda* (fall armyworm) adjust the relative abundance of specific troponin T mRNA transcripts in a precise, linear fashion to experimental manipulation of body weight (Marden et al., 2008). Skeletal muscle troponin T is a component of the troponin complex that regulates muscle contraction and force output (Brotto, 2005; Gomes et al., 2002; Ogut et al., 1999). Transcribed troponin T pre-mRNA is alternatively spliced, giving rise to multiple mature mRNA splice variants and, ultimately, protein isoforms, that affect skeletal muscle fiber force output in insects and mammals (Chandra et al., 2006; MacFarland et al., 2002; Marden et al., 1999, 2001). In insects, skeletal muscle troponin T is encoded by a single gene whereas in mammals there are two genes (i.e. slow and fast skeletal muscle *Tnnt1* and *Tnnt3*, respectively). In the rat, gastrocnemius muscle *Tnnt3* alternative splicing responds to natural growth and experimental manipulation of body weight (Schilder et al., 2011), in a similarly precise fashion to that reported for the fall armyworm moth flight muscles. Therefore, alternative splicing of troponin T appears to be one of the evolutionarily conserved mechanisms that can tune skeletal muscle sarcomere molecular composition in a quantitatively accurate manner to

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perceived variation in body weight. However, the detailed mechanics of how variation in body weight is indeed perceived and acted upon by skeletal muscle remain largely unknown.

We explored whether *Drosophila melanogaster* is a potential model system to examine such mechanisms. The *Drosophila* system has powerful genetic tools (reviewed in Wangler et al., 2017), including strain collections dedicated to genome-wide association mapping, genetic deficiency strains and several genetic manipulation tools (e.g. RNAi, Gal4-UAS and transposable element insertion tools), that could be leveraged to this purpose provided quantitative molecular adjustments to body weight variation occur in *Drosophila* skeletal muscles. To test this general hypothesis, we focused on *Drosophila* leg skeletal muscles. *Drosophila* leg muscles serve to support body weight under non-flying conditions, and are considered more analogous (as a result of their direct, synchronous nature; Eldred et al., 2010; Elliott and Sparrow, 2012) to vertebrate skeletal muscle than the indirect, asynchronous flight muscles. *Drosophila* skeletal muscle troponin T (encoded by the gene *upheld*, FlyBase ID Fbgn0004169; here, we will use its synonym *TnT* instead) exon structure is largely conserved with that of other insect and mammalian species, with, in addition to constitutive exons, a 5′ end alternatively spliced exon cassette and a mutually exclusive set of two 3′ end exons (i.e. exons 10a, 10b; Herranz, 2005). In the indirect flight muscles (IFM) and the jumping muscle (TDT; tergal depressor of trochanter), only two troponin T mRNA transcripts appear to be expressed (Benoist et al., 1998; Elliott et al., 2007), which contain none of the 5′ end alternatively spliced exons (see exon structure of *TnT_A* in Fig. 1) and differ only by whether 3′ end exon 10a or exon 10b is included (Herranz, 2005; Nongthomba et al., 2007). To our knowledge, the diversity of the troponin T transcript pool produced by alternative splicing in *Drosophila* leg skeletal muscles has not been examined.

To test whether leg muscle troponin T alternative splicing is sensitive to body weight variation in *Drosophila*, we used eight *Drosophila* genetic lines that vary in mean body mass. We first characterized troponin T alternative splicing in *Drosophila* leg muscles and then demonstrate that a 24 h exposure of leg muscles to increased body weight by means of hypergravity centrifugation results in significant, body weight-dependent changes in the relative abundance of troponin T transcripts, as well as enhanced leg muscle functional output.

MATERIALS AND METHODS

Flies

The following eight *Drosophila melanogaster* lines used in this study were obtained from the Bloomington Drosophila Stock Center at Indiana University: 25175 (RAL-301), 25177 (RAL-304), 25179 (RAL-307), 25189 (RAL-379), 25190 (RAL-380), 25199 (RAL-639), 25203 (RAL-732) and 25206 (RAL-786). The rationale for choosing these specific lines was that their mean body masses spanned the entire body mass range (0.49–0.77 mg, median: 0.6 mg; Jumbo-Lucioni et al., 2010) that was reported for 40 *D. melanogaster* lines that originally made up the *Drosophila* Genetic Reference Panel (DGRP; Ayroles et al., 2009; Mackay et al., 2005). This allowed us to study the quantitative *TnT* alternative splicing response to increased body weight across this naturally occurring body mass range. The DGRP resource is a much larger collection, currently 205 genetic lines (Huang et al., 2014).

Flies were reared on a standard diet (Nutri-Fly™ Bloomington formulation; Genesee Scientific, San Diego, CA, USA) at 23–24°C, under a 14 h/10 h dark/light regime, with the light period starting at 08:00 h. Stocks for line 25199 (RAL-639) were lost during our study and we therefore have no data on negative geotaxis and resting metabolic rate for this line (see Results). We used virgin male flies to avoid potential large-scale variability in the regulation of gene expression associated with egg development in females (Baker and Russell, 2009). Fly vials were cleared and newly emerged males separated from females into new vials the next morning, after which they matured for another 48 h. The age of all male flies used was between 72 and 80 h post-emergence (hpe).

Experimental manipulation of body weight

Manipulation of body weight was achieved using a custom-made, hypergravity centrifuge (Fig. 1), in a fashion similar to that described previously (Le Bourg, 2008). Briefly, a 2 horsepower, permanent magnet DC motor was connected to a rotating shaft by means of pulleys and a pulley belt. A 1 m diameter circular platform was attached to this shaft, allowing placement of fly vials along the edge of its surface. During hypergravity experiments, the whole platform (including fly vials) was enclosed with transparent vinyl sheeting to prevent significant desiccation of flies. Fly vials contained a thin (~0.5 cm) layer of diet medium and 20, 72–80 hpe virgin males of each *Drosophila* line, and were spun in the centrifuge at 12 ***g*** for 24 h. This level of gravity was chosen based on an observation from a previous study that fruit flies can 'hardly climb' under these conditions, whereas lower levels of gravity showed no apparent effects on locomotion (Herranz et al., 2008).

While 12 ***g*** may seem excessive, we can calculate that 12 ***g*** treatment of six-legged *Drosophila* is actually similar to the loading paradigm we previously used in four-legged rats (i.e. ~30% of unloaded body weight; Schilder et al., 2011). Ignoring most differences between endoskeletal and exoskeletal systems of invertebrates and vertebrates, when mass$^{2/3}$ scaling of muscle cross-sectional area needed to support body weight (e.g. Biewener, 1989) is assumed, we can estimate that, for *Drosophila*, the scaled equivalent of ~30% load on the leg muscle cross-section should be 17–25 times their body weight (Fig. S1). Extrapolation in this linear fashion indicates that exposure to 12 ***g*** may actually be relatively low in comparison to what rats were exposed to (Schilder et al., 2011). The above consideration notwithstanding, 12 ***g*** was also a technical limit of our experimental paradigm, as centrifugation of flies at larger ***g*** forces often resulted in fluid separating from the food medium, or food medium shifts, leading to drowning or crushing of flies residing in the lower regions of the fly vial during treatment. Control flies were not spun and were kept in vials next to the centrifuge. We determined the mean fly body mass for each vial of flies by measuring pooled fly mass to the nearest 0.1 mg, using a Mettler AE100 balance, and dividing this mass by the number of flies in the vial.

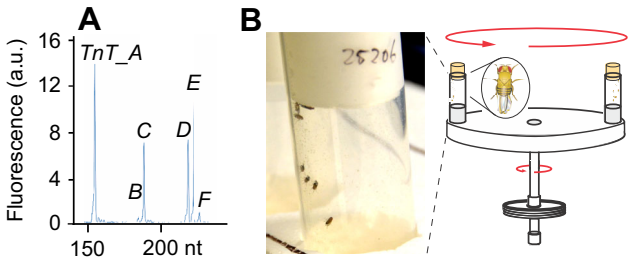

Fig. 1. Troponin T splice variants and experimental setup. (A) Sample trace from DNA fragment analyses of *D. melanogaster* 5′ troponin T gene (*TnT*) splice variants. (B) Right, hypergravity centrifuge illustration; left, generally observed fly positions post-24 h, 12 ***g*** treatment, i.e. flies aligned themselves along a relatively thin strip of the outer vial wall.

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TnT alternative splicing analysis

Flies were rapidly flash frozen in liquid nitrogen after treatment and total RNA was extracted from the frozen legs of 20 individuals from each line and treatment group using Trizol reagent (Invitrogen) followed by precipitation in isopropanol, according to the manufacturer's instructions. Legs were removed from fly thoraces using a small brush in a liquid nitrogen-chilled mortar cup. Total RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). *TnT* amplicons were amplified by PCR using fluorescein (FAM)-labeled forward primer dTnt_F1 (5′-FAM-GTTAGTCGAACCGCAG-CATT-3′), and an unlabeled reverse primer dTnt_R1 (5′-TGACGCTTGATGAACTCTGG-3′). Forward primer dTnt_F1 hybridizes to the *TnT* 5′-UTR, 99 nucleotides upstream of the *TnT* start codon. The mutually exclusive exon 10a/b at the 3′ end of *TnT* could potentially double the number of splice forms that could be generated from this gene. Our method does not currently allow for PCR amplicons larger than ∼1000 nucleotides to be accurately quantified (because of internal size standard limitations of the DNA fragment analysis protocol), which made a reverse primer location spanning these mutually exclusive exons impossible (i.e. the total amplicon size would be >1000 bp). Thus, while we refer to the *TnT* amplicons as splice variants throughout the manuscript, using our amplification strategy we could only quantify the variation in *TnT* alternative splicing derived from the 5′ end alternative splicing cassette.

PCR was performed using HotStart GoTaq polymerase (Promega, Madison, WI, USA) under the following cycling conditions: 5 min at 95°C, followed by 4 cycles of 30 s at 94°C, 30 s at 65°C (−1.0°C/cycle), followed by 30 s at 72°C. This was followed by 29 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C, ending with a final 15 min at 72°C. For quantitative analyses of *TnT* splice variant relative abundance in leg muscle samples, FAM-labeled PCR products were diluted 1:25 and 1 µl of this dilution was analyzed by capillary electrophoresis (ABI DNA Analyzer, Applied Biosystems). Any samples with a fragment peak height exceeding the linear detection range of the instrument were further diluted and run again. The relative abundance of each amplicon in the PCR reaction was determined by dividing its peak height by the total of all peak heights. Thus, our method to quantify alternative splicing events inherently standardizes to mRNA input amounts and normalizing to a reference gene is not necessary. Amplicon fragment size was determined using an internal size standard and Genemapper® (Applied Biosystems) fragment analysis software. Nucleotide sequences of all *D. melanogaster TnT* splice variants are deposited at NCBI (Benson et al., 2009; Sayers et al., 2009) and the *TnT* splice variants detected using the DNA fragment analysis showed exact size correspondence to these sequences (GenBank accession number: 665391163–665391182, 281360815).

TnT_A reaction norm

The presentation of *TnT* alternative splicing results is focused on observed effects of hypergravity on the relative abundance of the most abundant splice variant *TnT_A*, as changes in *TnT_A* relative abundance will by definition involve changes in the opposite direction of one or more other *TnT* splice variants. To allow a direct correlation of the effects of hypergravity on *TnT_A* relative abundance with fly body mass variation among lines, we defined the *TnT_A* reaction norm as the difference in relative abundance of the most abundant *TnT* splice variant *TnT_A* (Figs 3A and 1A) between leg muscles of control and experimental groups (for each of the eight lines).

Negative geotaxis

To examine effects of 24 h, 12 ***g*** treatment on leg muscle performance (i.e. walking/running speed), a variation on the rapid iterative negative geotaxis method (Gargano et al., 2005) was used on three sets of flies independent from those sets examined for 12 ***g*** effects on leg *TnT* alternative splicing, and jump performance and resting metabolic rate (see below). The experiment was started 30 min after the 12 ***g*** treatment was completed. Briefly, fly vials (four at a time) containing approximately 20 flies were dropped onto a straight surface and flies (now all at the bottom of the vial) were allowed to climb up the vial for 3 s, at which point a digital photograph was taken. Vials were placed in front of an LED backlit screen to provide sufficient contrast for photography. We repeated this procedure two times for each set of four vials in quick succession (i.e. trials lasted about 15 s). In total, negative geotaxis was determined for flies in 42 vials (i.e. seven lines, two treatments, in triplicate) in this fashion. From the resulting digital images, we determined the mean percentage of individual flies located in the top quadrant of the vial (i.e. between 75% and 100% of total available vial height) across the two replicates, using ImageJ software. In a fashion similar to *TnT_A* relative abundance data, we defined the reaction norm of negative geotaxis as the difference in the mean number of individuals in the top quadrant of the vial between control and experimental groups for each line, to allow us to directly correlate this response with fly body mass variation.

Jump performance

In flies from an independent 12 ***g*** experiment, we examined fly jump performance as an additional test for 12 ***g*** effects on leg performance. Preparation of control and 12 ***g***-treated flies for assessment of jump performance was started 30 min after 12 ***g*** treatment using a method similar to that described previously (Zumstein et al., 2004). Briefly, flies were anesthetized with CO_2 before clipping their wings with micro-scissors, after which they were given another 30 min to recover from CO_2 anesthesia. Individual flies were then released onto a large white paper sheet and stimulated to jump by moving by hand a relatively large black looming object (cap of a black Sharpie pen) towards the rear of the flies. Care was taken not to touch the flies and jumping attempts were recorded at 60 frames s^{-1} using a CASIO Exilim ZR-100 camera mounted vertically above the paper sheet by means of a tripod. For five flies from control and 12 ***g*** groups from each of the eight lines (note that we accidentally lost two individuals during experiments), we recorded the first three jumps attempted in this fashion and determined mean jump distance using the open source Tracker software (http://physlets.org/tracker/) to track fly position from individual video frames. To avoid excessive handling and potential damage, we did not collect body masses prior to the experiments, nor was it possible to do so for wingless flies after the experiment.

Resting metabolic rate

Resting metabolic rate was determined using flow-through respirometry performed in a benchtop incubator maintained at 24°C. CO_2-free, dry air produced by a Whatman FT-IR Purged Gas Generator was pumped through 30 ml respirometry chambers via a Sable Systems Respirometer Multiplexer V2.0 system at 125 ml min^{-1}. Chambers were plugged with cotton to prevent flies from escaping. Respirometry was performed in complete darkness to minimize fly activity. Using a LI-COR 6262 (LI-COR Biosciences, Lincoln, NE, USA) carbon dioxide analyzer, we obtained CO_2 exchange rate measurements from seven experimental

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chambers relative to an empty control chamber, which was re-sampled prior to every experimental chamber measurement so that any baseline drift could be removed in post-processing of data. In addition to CO_2 exchange rate, flow rate and ambient temperature were recorded using mass flow controller units and a thermocouple, respectively, and collected with a UI2 data acquisition system controlled by ExpeData (Sable Systems, Las Vegas, NV, USA) software. Animal chambers were measured for 400 s each while the empty control chamber was measured for 225 s each time. Mean net $\dot{V}_{CO_2}$ (ml CO_2 h^{-1}) during these measurements was calculated from raw CO_2 ppm data using custom IGOR Pro (Wavemetrics, Inc., Lake Oswego, OR, USA) scripts. We used groups of 3–5 males from each of seven lines (the culture of line 25199 was lost prior to these experiments) and treatment groups (control and 12 ***g***) and used mean body mass for each of these groups to calculate mass-specific $\dot{V}_{CO_2}$ (i.e. ml CO_2 h^{-1} g^{-1} fly). Measurements were started 45 min after the end of the 12 ***g*** experiments and experiments were repeated twice for each line.

Statistics

No formal *a priori* statistical power analysis was performed, but rather sample size requirements for *TnT* alternative splicing analyses were estimated on the basis of a preliminary study, using line 25175, of effects of 12 ***g*** on leg muscle *TnT_A* relative abundance, jump performance, resting metabolic rate and negative geotaxis, which showed high replicability of results for the first three traits, and higher variability in the last. For statistical analyses, *TnT* splice variant relative abundance was first arcsine transformed to allow linear model fitting. A principal component analysis was used to reduce the *TnT* splice variant relative abundance dataset to two orthogonal, principal axes of variation. We used two-way ANOVA (with interaction terms) to examine the effects of 12 ***g*** treatment and fly line on *TnT* alternative splicing, negative geotaxis, jump performance and resting metabolic rate. In addition to providing statistical details in the text, in Figs 2 and 4–6, we present the results of statistical analyses as least squares means (showing main effects of hypergravity after taking into account effects of fly line (and/or other factors), as well as providing actual means for all lines.

RESULTS

TnT splice variant identification

We detected six *TnT* 5′ splice variants in male *D. melanogaster* leg samples (Table 1), the largest one of which (i.e. a 225 nucleotide amplicon using our PCR strategy), *TnT_F*, was consistently expressed in leg muscles but at very low levels (i.e. typically <1%; Fig. 1A). Fig. 1 shows the relationship between fluorescently labeled fragment sizes determined by the ABI DNA Analyzer and predicted splice variant sequences from deposited *Drosophila TnT* mRNA sequences. Interestingly, splice variants *TnT_E* and *TnT_F* appear to contain a micro-exon located between coding exons 1 and 3. Note that this observation is based on manually aligning deposited *TnT* mRNA sequences for *Drosophila*; we did not confirm this observation by Sanger sequencing. Previously another micro-exon was reported between exon 3 and 5 (i.e. coding exon 4 in fig. 1B of Benoist et al., 1998), but while the micro-exon in question here is apparent from the 18 deposited *TnT* mRNA transcript sequences at GenBank (accession numbers 665391163–665391182, 281360815), to our knowledge the expression of a micro-exon between *TnT* coding exon 1 and 3 in tissues has not been reported previously. It is very likely that inclusion of this second *TnT* micro-exon is specific to the leg musculature, given that previous studies on *Drosophila* muscle *TnT* alternative splicing did not include leg muscles (Benoist et al., 1998; Nongthomba et al., 2007).

Behavioral observations post-centrifugation

Obtaining video footage of flies undergoing the 12 ***g*** treatment was not feasible, but we did observe fly position and behavior immediately post-experimental treatment. In all experiments, centrifuged flies had positioned themselves along a narrow strip of the vial wall (Fig. 1B) furthest removed from the center of the centrifuge and their legs were therefore exposed to a force vector of approximately similar magnitude and direction. Flies also appeared to have adopted a somewhat crouched position, with their legs in a more flexed state than under normal gravity, suggesting they had indeed undergone loading at a level that prevented them from walking around in the vial. While we did not quantify this change in position, we concluded that treated flies experienced a constant load throughout the 24 h treatment. Flies stayed fixed in position for approximately 20–30 s after the centrifuge had stopped spinning, after which they initiated normal walking, flying and feeding behavior.

Effect of hypergravity on leg muscle *TnT* alternative splicing

Experimental body weight increase following 24 h exposure to 12 ***g*** resulted in significant changes to the overall mixture of *TnT* splice variants in leg muscles of male flies from all eight lines, as subjecting values for the first principal component (PC1) to a two-way ANOVA with factors fly line, treatment and fly line×treatment yielded significant main effects for fly line and treatment, but not for the interaction term (Table 2). At the individual *TnT* splice variant level, two-way ANOVA indicated a significant decrease in the relative abundance of the most abundant *TnT* splice variant in leg muscle, *TnT_A* (Table 2, Fig. 2A), which was consistent across the eight lines (Table 2, Fig. 2B).

The mean *TnT_A* reaction norm correlated significantly (R^2=0.772, P=0.0040; Fig. 3) with mean fly line body mass across two independent experiments; that is, heavier fly lines

Table 1. Predicted translated exon structure for leg muscle *TnT* amplicons identified in this study

	Coding exon					
TnT splice variant	1	2	3	4	5	6
TnT_A	MSDDEEYT					GEGDPEFIKR
TnT_B	MSDDEEYT		SEEEEVVEETREET			GEGDPEFIKR
TnT_C	MSDDEEYT		SEEEEVVEETREET	K		GEGDPEFIKR
TnT_D	MSDDEEYT		SEEEEVVEETREET	K	KPPQTPAE	GEGDPEFIKR
TnT_E	MSDDEEYT	S	SEEEEVVEETREET		KPPQTPAE	GEGDPEFIKR
TnT_F	MSDDEEYT	S	SEEEEVVEETREET	K	KPPQTPAE	GEGDPEFIKR

Translations are based on comparisons of *TnT* amplicon sizes determined by DNA fragment analysis (see Materials and methods) with an alignment of all available *D. melanogaster TnT (upheld)* GenBank sequences.

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Table 2. *F*-statistics and associated *P*-values for effects of fly line, hypergravity treatment and their interaction on *Drosophila melanogaster* leg muscle *TnT* splice variant relative abundance

TnT splice variant	Line		Treatment		Line×treatment	
	F	*P*	*F*	*P*	*F*	*P*
TnT_A	31.58	<0.0001	27.94	<0.0001	0.69	0.68
TnT_B	9.94	0.0002	2.30	0.15	0.10	0.997
TnT_C	19.95	<0.0001	1.44	0.25	0.34	0.92
TnT_D	23.90	<0.0001	12.08	0.003	0.37	0.91
TnT_E	7.57	0.0007	12.66	0.003	0.24	0.97
TnT_F	4.04	0.01	13.97	0.002	1.87	0.14
PC1	17.94	<0.0001	19.63	0.0006	0.56	0.78

Results of two-way ANOVA (N=32, α=0.05) predicting the effects of fly line and hypergravity (12 ***g***) treatment on the relative abundance of individual *TnT* splice variants. The Bonferroni adjusted *P*-value cutoff is 0.003 (which is rather strict, but emphasizes the significance of 12 ***g*** treatment effects on *TnT_A* abundance). Bottom row shows how fly line and treatment are significant predictors of the first principal component (PC1) of overall variation in leg muscle *TnT* splice variant relative abundance. Significant fly line×treatment interactions were not observed. *TnT* splice variant relative abundance was arcsine transformed prior to model fitting.

showed a greater decrease in *TnT_A* relative abundance. This indicates that the effect of 12 ***g*** on *TnT* alternative splicing depended on the actual weight load experienced by *Drosophila* leg muscles rather than consisting of more random quantitative changes in the same direction (i.e. a decrease in *TnT_A* splice variant abundance in experimental flies). These findings provide further support for the conclusions regarding the body weight dependency of *TnT* alternative splicing observed in flight muscles of *Spodoptera* moths (Marden et al., 2008) and gastrocnemius muscle in *Rattus norvegicus* (Schilder et al., 2011).

Negative geotaxis

To examine whether the observed changes in *TnT* alternative splicing in response to 12 ***g*** treatment were associated with altered leg muscle function, we measured negative geotaxis responses of three independent sets of flies from each line and treatment group. We scored the percentage of individuals in the top quadrant of total available vial height, averaged those values across the two trials we performed, and subjected these average scores to a two-way ANOVA with interaction term using fly line and treatment as factors, and replicate as a random factor (i.e. we performed three replicate experiments for each line under control or 12 ***g*** treatment; see Materials and methods). This analysis yielded a significant main effect for treatment ($F_{1,42}$=6.92, P=0.014; Fig. 4A), such that the percentage of individuals in the top vial height quadrant was generally higher for 12 ***g***-treated lines (17.3±0.01%, mean±s.e.m.) than for control lines (12.3±0.01%). The effect of fly line was also significant ($F_{6,42}$=8.73, P<0.0001). Moreover, the interaction effect of fly line and treatment was significant ($F_{6,42}$=2.63, P=0.04), indicating that the effect of 12 ***g*** on negative geotaxis was line dependent. Indeed, Fig. 4B illustrates that lines 25189, 25190 and 25203 did not show the increase in the mean percentage of individuals in the top quadrant in response to 12 ***g*** that the other lines did. While the reaction norm for negative geotaxis did not show a statistically significant correlation with mean body mass for each line (across the three replicate experiments), a similar trend (R^2=0.545, P=0.058; Fig. 4C) to that of the *TnT_A* reaction norm was observed (Fig. 4C). However, this trend strongly depended on results for line 25177, which had a mean body mass in this experiment that was significantly greater than that of the flies used for the *TnT_A* reaction norm analyses, and was no longer apparent when data for this line were excluded (R^2=0.127, P=0.487; data not shown).

Jump performance

It was unclear whether the effect of 12 ***g*** treatment on leg muscle *TnT* alternative splicing (Fig. 2) was present in all, or a subset of muscles of the legs, as we dissected the entire set of six legs from each individual and extracted RNA from the total amount of legs from 20 individuals. A major contribution to the total leg musculature is made by the trochanter depressor and levator muscles (Soler et al. 2004), but of note here is that in the mesothoracic leg pair the trochanter depressor muscle is an enlarged muscle (TDT) with a tergal origin (i.e. it attaches to the ventral side of the dorsal thoracic cuticle). This muscle is mostly involved in the jump behavior of *Drosophila* (Elliott and Sparrow, 2012; Elliott et al., 2007), whereby contractions of the two TDT muscles extend the mesothoracic pair of legs to initiate a jump. Flexion of the legs in response to the forces experienced under 12 ***g*** centrifugation (as our observations post-experiment indicate) likely stretched (or was resisted by) these muscles in a manner quantitatively equivalent to the body weight experienced under centrifugation conditions. Our leg muscle tissue samples likely included a significant portion of the TDT muscles and the effect we observed for *TnT* alternative splicing (Fig. 2, Table 2) therefore may have extended to the entire TDT muscles in the thorax. Moreover, mutations that impair normal *TnT* alternative splicing in *Drosophila* TDT muscles can have significant effects on jump performance (Elliott et al., 2007). Therefore, we hypothesized that jump performance is affected by 12 ***g*** treatment.

Measures of jump distance (see Materials and methods) were subjected to a two-way ANOVA with interaction term using fly line and treatment as factors. Overall, there was much less variation in

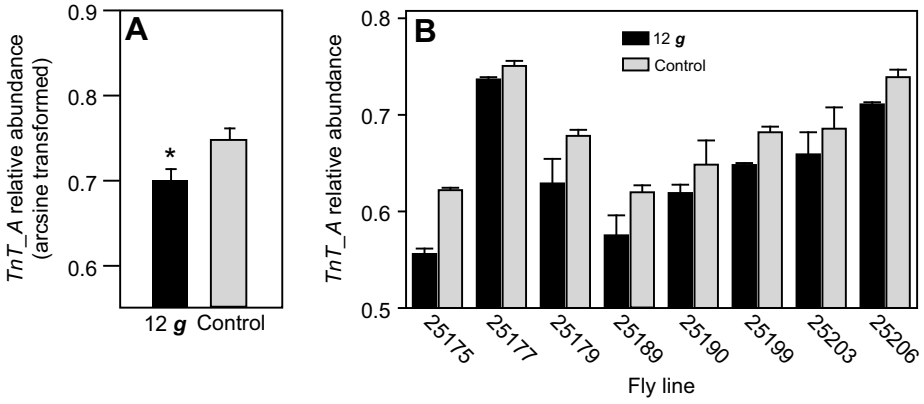

Fig. 2. Hypergravity treatment causes significant changes to *D. melanogaster* leg muscle *TnT* alternative splicing. (A) Least squares means estimated by linear modeling (2-way ANOVA, total N=32, N=16 per treatment) of arcsine-transformed *TnT_A* relative abundance, illustrating the main effect of 12 ***g*** treatment (after adjusting for effects of fly line). Asterisk indicates a significant difference. (B) Actual mean (non-transformed) relative abundance of *TnT_A* for each fly line and treatment. Error bars represent s.e.m. (N=2 per treatment group).

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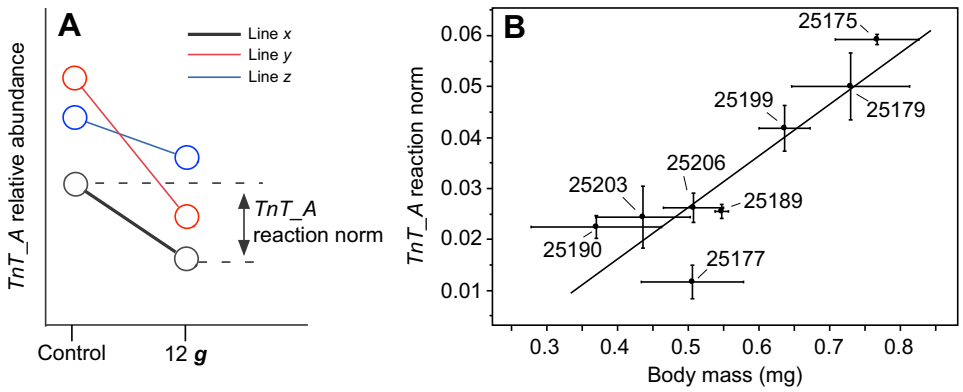

Fig. 3. *TnT_A* reaction norm and body mass. (A) Illustration of the method (see Materials and methods) used to calculate the *TnT_A* reaction norm for three imaginary fly lines exposed to control and 12 ***g*** treatments. (B) Mean *TnT_A* reaction norm for eight fly lines plotted against their mean body mass. Linear fitting (see Results for R^2) was performed on the eight mean values. Body mass means were determined from all individuals (i.e. control and 12 ***g***-treated groups combined) of a given fly line. Line designations are indicated for clarity. Error bars represent s.e.m. (*N*=4 groups of 20 per fly line for body mass means, and *N*=2 groups of 20 per fly line for *TnT_A* reaction norm means). See also Table 3.

jump distance among the lines (except line 25199, which performed poorly compared with the other lines) than observed in the negative geotaxis experiments. There were significant main effects of fly line ($F_{7,78}$=21.3, P<0.0001) and treatment ($F_{1,78}$=13.68, P=0.0005), such that the jump distance in 12 ***g***-treated flies was higher (2.6±0.057 cm, mean±s.e.m.) than for control lines (2.2±0.059 cm; Fig. 5A). The interaction term was not significant ($F_{7,78}$=1.65, P=0.137), indicating that the fly lines did not significantly differ in their jump distance response to 12 ***g***. However, Fig. 5B illustrates that 12 ***g*** treatment did not affect the mean jump distance in lines 25189, 25190 and 25199.

Resting metabolic rate

To examine whether the 12 ***g*** treatment was a potential stressor to flies that could have resulted in the observed overall increased jump performance and/or negative geotaxis responses, we determined resting metabolic rate in groups of control and treated flies (Fig. 6A). These analyses were performed on 7 out of the 8 original lines, because the culture of line 25199 was lost prior to this set of experiments. Mass-specific CO_2 exchange rates ($\dot{V}_{CO_2}$, in ml CO_2 h^{-1} mg^{-1} fly) were subjected to a two-way ANOVA with interaction term, using fly line and treatment as main factors. There was a significant main effect of line ($F_{6,28}$=4.36, P=0.011) but not

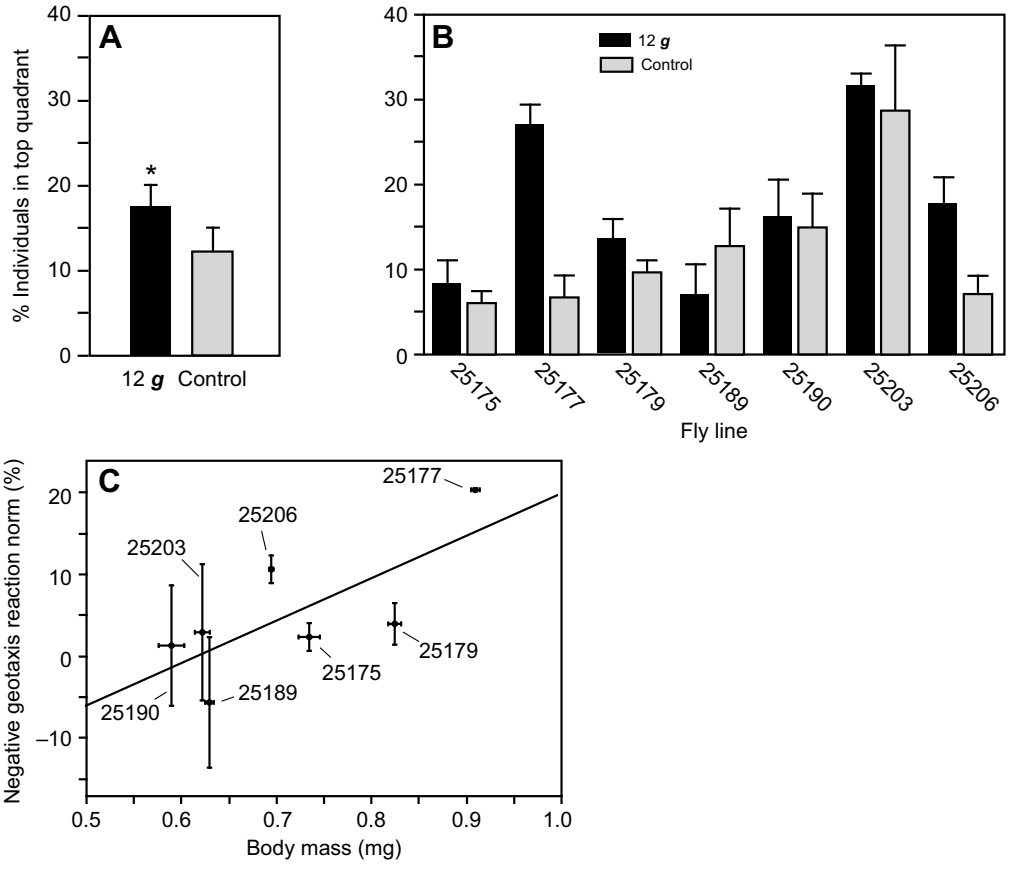

Fig. 4. Hypergravity treatment significantly increases overall mean negative geotaxis responses across the eight *D. melanogaster* fly lines used. (A) Least squares means estimated by linear modeling (2-way ANOVA, total *N*=42 vials measured, *N*=3 vial means per treatment group; see Results for details) of negative geotaxis data (i.e. percentage of flies residing in the top quadrant of the vial), illustrating the main effect of 12 ***g*** treatment (after adjusting for effects of fly line). Asterisk indicates a significant difference. (B) Actual mean (non-transformed) percentages for each fly line and treatment. Error bars represent s.e.m. (C) Mean negative geotaxis reaction norm plotted against mean fly line body mass, determined for all individuals (i.e. control and 12 ***g***-treated groups combined) of a given fly line. Fly line identifications are indicated for clarity; note that data for fly line 25199 are missing (see Materials and methods). Error bars represent s.e.m. (*N*=6 vial means per line for body mass means, *N*=3 vial means per line for negative geotaxis reaction norm means).

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Table 3. Body mass means±s.e.m. for each *D. melanogaster* line examined for effects of hypergravity on leg muscle *TnT* alternative splicing

D. melanogaster line	Body mass (mg)
25175	7.68±0.6
25177	5.06±0.7
25179	7.30±0.8
25189	5.48±0.1
25190	3.70±0.9
25199	6.37±0.4
25203	4.36±0.7
25206	5.08±0.4

Effects of hypergravity (24 h, 12 ***g***) treatment were examined on *N*=4 groups of 20 males (see Fig. 3).

of treatment ($F_{1,28}$=0.002, P=0.97) or the interaction term ($F_{6,28}$=2.36, P=0.09), indicating that resting metabolic rate did not differ between control and 12 ***g***-treated lines. Fig. 6B,C further illustrates this statistical result, showing no obvious pattern in mean mass-specific CO_2 exchange rates for control and experimental groups among lines.

DISCUSSION

The effects of hypergravity (~1–7.5 ***g***) treatment have previously been examined for several *Drosophila* traits, including egg viability, longevity, response to stressors and climbing activity (Le Bourg, 2008). These levels of hypergravity are considered a mild stressor that, even though actual metabolic rate under such conditions has not been measured, is assumed to increase metabolic demand (i.e. as fly body weight increases, more energy needs to be spent to support and move this extra weight). Hypergravity centrifugation has also been employed to increase body weight in rats, and was demonstrated to affect body mass gain in a dose-dependent manner (i.e. exposure to 1, 1.5, 2.0, 2.5 and 3.5 ***g*** caused reduced body mass gain in an incremental fashion; Kita et al., 2006) during 14 day trials, suggesting sensitivity to quantitative variation in body weight for this trait. Pertinent to the focus of the current study, hypergravity was also demonstrated to affect rat skeletal muscle sarcomere molecular composition (Bozzo, 2004; Stevens et al., 2003). The reported effects in these studies were more qualitative in nature (i.e. sarcomere protein levels at 1 versus 2 ***g***) and complicated by parallel reductions of body mass and/or increases in skeletal muscle mass, but nonetheless demonstrate the utility of the centrifugation method (which is not complicated by backpacks or locally applied weight, but rather lets the whole body experience a varying gravity vector) in the study of body weight sensitivity of skeletal muscle molecular plasticity.

Here, we demonstrated that short-term (24 h) experimental increases of body weight using hypergravity (12 ***g***) centrifugation resulted in significant and changes to *Drosophila* leg muscle *TnT* alternative splicing, indicating that flies can sense their body weight and adjust leg muscle composition at a molecular level to variation in body weight. Moreover, our results indicate that this adjustment is quantitatively accurate as the *TnT* alternative splicing response (i.e. *TnT_A* reaction norm) showed significant positive correlation with fly body weight (Fig. 3) and, therefore, with total absolute load experienced by the leg muscles. We have thus provided additional support for the claim that body weight sensitivity of *TnT* alternative splicing in muscles involved in body weight support is an evolutionarily conserved trait in animals (Schilder et al., 2011). Effects of 12 ***g*** treatment on the performance of leg muscles were also evident, as 12 ***g***-treated flies generally showed increased negative geotaxis responses (Fig. 4) and voluntary escape jump performance (Fig. 5). These changes to whole-organismal locomotor performance traits did not appear to be due to 12 ***g*** treatment-induced stress, as indicated by a lack of effect of 12 ***g*** treatment on resting metabolic rate (Fig. 6).

When we compare the effect of body weight increase on *TnT* alternative splicing in *Drosophila* with that observed in other organisms, an interesting pattern emerges. The 12 ***g***-induced reduction in *Drosophila* leg muscle *TnT_A*, the most abundant and smallest splice variant (Fig. 1A), is similar to findings by Marden et al. (2008). That study demonstrated that the relative abundance of the most abundant, smallest troponin T splice variant (*Tnt_F*) in the flight muscles of the fall army worm *S. frugiperda*, negatively correlates with body weight variation caused by weight-loading interventions. However, other work (Marden et al., 1999, 2001) and unpublished findings from our laboratory showed that in dragonfly (*Libellula pulchella*) flight muscles, one of two highly abundant splice variants, *Tnt 261*, correlates positively with body weight variation. Similarly, for rat (*Rattus norvegicus*) gastrocnemius muscle, the most abundant fast skeletal muscle troponin T splice variant, *Tnnt3 a1*, is positively correlated with body weight increase due to natural growth and experimental manipulation (Schilder et al., 2011). Thus, these molecular responses to body weight increase typically involve the most abundantly expressed troponin T splice variants in insects and mammals. And, even though the exact nature of the response (i.e. negative versus positive correlations) appears to vary somewhat among invertebrates and mammals, for both *L. pulchella* and *R. norvegicus*, they are predicted to enhance muscle fiber calcium sensitivity of force output (Brotto, 2005; Ogut et al., 1999) and whole-muscle performance (Marden et al., 2001). These functional relationships have yet to be demonstrated in *Spodoptera* and

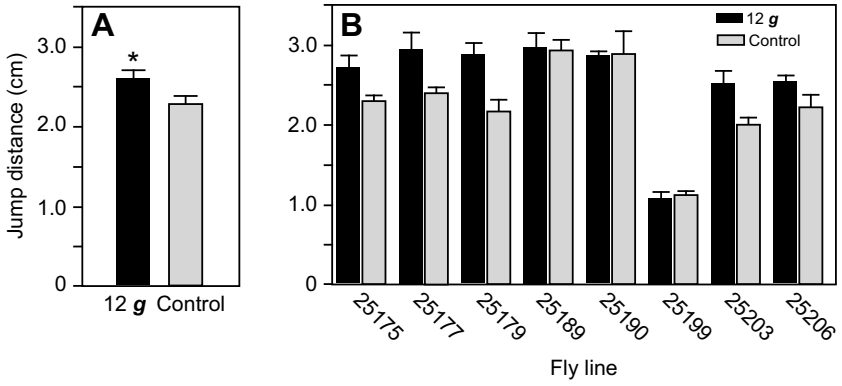

Fig. 5. Jump distance is significantly increased in response to hypergravity treatment across the eight *D. melanogaster* fly lines used. (A) Least squares means estimated by linear modeling (two-way ANOVA, *N*=78; see Results for details) of jump distance, illustrating the main effect of 12 ***g*** treatment (after adjusting for effects of fly line). Asterisk indicates a significant difference. (B) Actual mean jump distance for each line and treatment used in the linear model. Error bars represent s.e.m. (*N*=5 males per treatment group, except for 12 ***g***-treated 25190, and control 25199 groups, where *N*=4 males).

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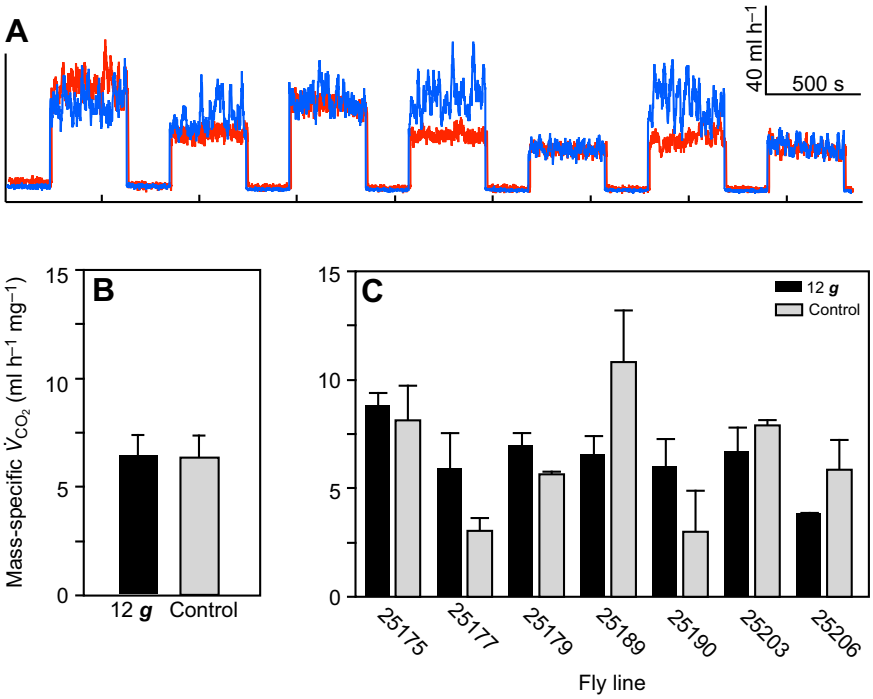

Fig. 6. Mass-specific resting metabolic rate ($\dot{V}_{CO_2}$) is unaffected by hypergravity treatment. (A) Sample $\dot{V}_{CO_2}$ traces obtained for control (blue trace) and experimental (red trace) groups of five males from seven *D. melanogaster* lines. The square-wave appearance of the trace is due to the animal chambers and reference chamber being measured in sequence. Note that these traces have not yet been normalized to mean fly body mass in each of the respirometer chambers. Thus, the *y*-axis represents $\dot{V}_{CO_2}$ in ml h^{-1}, whereas the *x*-axis represents time in s. (B) Least squares means estimated by linear modeling (2-way ANOVA, total *N*=28; see Results for details) of mass-specific resting metabolic rate (after adjusting for effects of fly line). (C) Actual mass-specific resting metabolic rate for each line and treatment used in the linear model; note that data for line 25199 are missing (see Materials and methods). Error bars represent s.e.m. (*N*=4 per line, *N*=2 per treatment).

Drosophila, although impaired regulation of *TnT* alternative splicing in the *Drosophila* TDT muscles has been demonstrated to negatively affect jump force output (Elliott et al., 2007).

An open question for all systems investigated is whether the observed *TnT* response is due specifically to mechanical loading of the leg muscles (or, in the case of *Spodoptera*, flight muscles), or whether the increased body weight is sensed (centrally or peripherally), and muscle plasticity controlled by some systemic factor(s). To our knowledge, there is no information available on this latter possibility, but available literature suggests that there are skeletal muscle-inherent force sensors (e.g. Ingber, 1997; Jaalouk and Lammerding, 2009; Linke and Krüger, 2010; Miller et al., 2004) that control mechanisms involved in muscle plasticity. Our work on cultured C2C12 myotubes (Schilder et al., 2012) demonstrated that alternative splicing of troponin T mRNA in isolated muscle cells responds to loading by means of cyclic stretching in a fashion similar to that observed in adult rats (Schilder et al., 2011), providing additional support for the hypothesis that muscle-inherent sensors and effectors are involved. While to our knowledge this is unknown in vertebrates, in invertebrates (e.g. cockroaches, stick insects) there is significant evidence that there are neural sensors (e.g. campaniform sensillae) whose activity encodes loads experienced by legs in a quantitative manner (Noah et al., 2004; Zill et al., 2004, 2017). These sensors have been mostly studied with regard to shorter term control of posture and locomotion, and it therefore remains unknown whether, or how, longer term exposure to (body weight-dependent) activity of such sensors may affect leg muscle composition.

As we could not record fly behavior in the vials during the hypergravity trials, it is unclear whether experimental groups of flies were capable of feeding during the 24 h period. It is therefore possible that observed effects on *TnT* alternative splicing could have

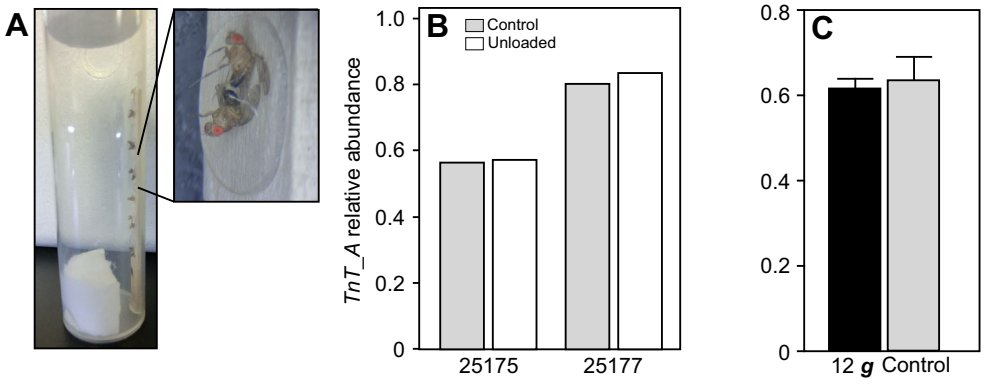

Fig. 7. *Drosophila* body weight sensor location. (A) Hypergravity treatment (24 h, 12 ***g***) did not appear to affect leg muscle *TnT* alterative splicing in male flies whose legs were not supporting body weight ('unloaded', see B). Flies were attached dorsally (see Discussion) so that their legs were suspended. Moist cotton was provided to prevent desiccation. (B) *TnT_A* relative abundance in legs from control and unloaded flies. This experiment was performed once using two of the original eight DGRP lines, hence the absence of a standard error bar (i.e. *N*=1/treatment). (C) Hypergravity treatment (24 h, 12 ***g***) did not appear to affect whole-thorax muscle *TnT* alternative splicing in fly line 25175 males (*N*=2/treatment). Because of low sample sizes, no formal statistical analyses were performed on these data.

been affected or caused by food deprivation. Wild-type *D. melanogaster* were previously shown to primarily engage in feeding behavior during the first 4 h of daily light periods (Seay and Thummel, 2011). In our studies, hypergravity experiments started between 12:00 h and 13:00 h, so if the DGRP lines investigated here follow the same circadian feeding rhythm, flies would have been food deprived for only a few hours at the end of a given experiment. However, preliminary results from a study of the effects of 12 ***g*** hypergravity on flies whose legs were not supporting body weight (see below; Fig. 7), and therefore definitely did not feed during the experiment, showed a lack of effect of 12 ***g*** treatment on *TnT* alternative splicing, indicating that food deprivation had little effect on this mechanism. Moreover, Marden et al. (2008) showed that food deprivation in the fall army worm (*S. frugiperda*) caused an increase in relative abundance of the most abundant *TnT* splice variant compared with fed controls, rather than the decrease in relative abundance that we observe in hypergravity-treated flies. Interactions of dietary history with skeletal muscle plasticity mechanisms are the subject of ongoing research in our laboratory (e.g. Schilder et al., 2011), and our experimental paradigm can be readily leveraged to examine effects of diet on leg muscle *TnT* alternative splicing in *D. melanogaster*. As the 24 h hypergravity treatment appears to force flies into a crouched position with little apparent ability to move, it will be equally interesting to determine whether more dynamic leg muscle loading, as occurs during chronic exercise (e.g. *sensu* Mendez et al., 2016; Piazza et al., 2009), will affect *TnT* alternative splicing in a similar, body weight-dependent manner.

To start an examination of the location of a potential body weight sensor in *Drosophila* leg muscles, we performed an experiment in which males were glued dorsally to a lolly stick (using Aleene's® tacky dots singles™; Fig. 7A), and subsequently exposed to 24 h, 12 ***g*** centrifugation. Thus, while their whole bodies were exposed to 12 ***g***, their legs were not supporting the increased body weight. Results from this experiment indicated there is no effect of 12 ***g*** treatment on leg muscle *TnT* alternative splicing in these males (Fig. 7B), and that the sensor is inherent to the legs (muscle or nervous system). Results from another study (Fig. 7C) showed that *Drosophila* thoracic muscle *TnT* alternative splicing was not sensitive to 24 h, 12 ***g*** hypergravity treatment, again suggesting that the observed effects were specific to body weight-supporting leg muscles. Note, however, that these results comprise a very limited dataset currently, i.e. data in Fig. 7A,B were obtained for line 25175 and 25177 only, and data in Fig. 7C for line 25175 only, and will require replication and validation in the other lines.

In contrast with the *TnT_A* reaction norm results (Fig. 3B), the negative geotaxis reaction norm was not significantly positively correlated with mean fly body mass (Fig. 4C). While we were unable to determine this relationship for jump performance in this study (see Materials and methods), the potential for a correlation between mean fly body mass and jump distance response is not apparent from the data presented in Fig. 5 (i.e. three lines showed no response, while the others showed a very similar response). It is possible that the 12 ***g*** treatment we imposed on flies affected *TnT* mRNA levels, but was not of sufficient length (i.e. only 24 h) for protein expression changes to have been fully implemented, and affect walking and/or jump ability. While *TnT_A* (the most abundant mRNA in the expressed *TnT* pool; Fig. 1A) is presumably translated to the respective protein isoform, we did not measure protein expression in this study to address this possibility. Alternatively, negative geotaxis behavior (and possibly jump performance) in *Drosophila* may generally correlate poorly with body mass, and the molecular composition of body weight support muscles.

Finally, even though no hypergravity-induced stress was indicated by our measurements of resting metabolic rate, it is possible that the 12 ***g*** treatment caused other physiological or neurological effects on the ability or motivation to perform in negative geotaxis trials via mechanisms independent of leg skeletal muscle molecular composition. Such a scenario could also explain the lack of observed correlation of negative geotaxis and mean body mass similar to that observed for the *TnT_A* reaction norm. Overall, a mechanistic link between 12 ***g***-induced changes to *TnT* alternative splicing, molecular composition of the leg muscles and *Drosophila* locomotor function therefore has to remain somewhat speculative.

To remedy this, it is crucial that we start assessing the relationships between body weight, *TnT* alternative splicing and muscle function more directly and in a standardized fashion that is unaffected by motivation to perform a particular behavior. The TDT and T2 tibial levator muscles (the latter of which are also activated during giant axon activation-induced jumps; Trimarchi and Schneidermann 1993) may be good model muscles to pursue this goal. Static calcium sensitivity of muscle fiber force output and muscle dynamic mechanical properties (e.g. shortening velocity) of *Drosophila* TDT muscles can be readily tested in *in vitro* settings (Eldred et al., 2010; Koppes et al., 2014) and it is feasible to accurately assess maximum jump force output in response to experimental stimulation (and/or local bisection) of the giant axon system that innervates it (Elliott and Sparrow, 2012). Further evaluation (e.g. varying the exposure time, examining sarcomere protein expression level effects) of our hypergravity centrifugation paradigm, combined with these *in vitro* leg muscle function assays and *Drosophila*'s extensive genetics resources will allow us to more directly and mechanistically link body weight-dependent molecular plasticity of skeletal muscles to relevant functional outcomes. Importantly, this will set the stage for studies aimed at identifying and locating the body weight sensor that communicates with mechanisms controlling this aspect of skeletal muscle plasticity, and how this communication (dys)functions in disease phenotypes in which proper tuning of the molecular composition of skeletal muscles to body weight is failing, as previously demonstrated for obese rats (Schilder et al., 2011).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: R.J.S.; Methodology: R.J.S.; Formal analysis: R.J.S., M.R.; Data curation: R.J.S., M.R.; Writing - original draft: R.J.S.; Writing - review & editing: R.J.S.; Supervision: R.J.S.; Project administration: R.J.S.

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