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Weight and nutrition affect pre-mRNA splicing of a muscle gene associated with performance, energetics and life history

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SUMMARY

A fundamental feature of gene expression in multicellular organisms is the production of distinct transcripts from single genes by alternative splicing (AS), which amplifies protein and functional diversity. In spite of the likely consequences for organismal biology, little is known about how AS varies among individuals or responds to body condition, environmental variation or extracellular signals in general. Here we show that evolutionarily conserved AS of *troponin-t* in flight muscle of adult moths responds in a quantitative fashion to experimental manipulation of larval nutrition and adult body weight. *Troponin-t* (*Tnt*) isoform composition is known to affect muscle force and power output in other animals, and is shown here to be associated with the thorax mass-specific rate of energy consumption during flight. Loading of adults with external weights for 5 days caused an AS response nearly identical to equal increases in actual body weight. In addition, there were effects of larval feeding history on adult *Tnt* isoform composition that were independent of body weight, with moths from poorer larval feeding regimes producing isoform profiles associated with reduced muscle performance and energy consumption rate. Thus, *Tnt* isoform composition in striated muscle is responsive to both weight-sensing and nutrition-sensing mechanisms, with consequent effects on function. In free-living butterflies, *Tnt* isoform composition was also associated with activity level and very strongly with the rate of egg production. Overall, these results show that AS of a muscle gene responds in a quantitative fashion to whole-organism variables, which apparently serves to coordinate muscle strength and energy expenditure with body condition and life history.

Key words: feeding history, phenotypic plasticity, body condition, weight sensing, nutrient sensing, metabolic rate, muscle performance, oogenesis, alternative splicing.

INTRODUCTION

Much of animal energy expenditure is due to muscle activity, which is regulated primarily by behavior (e.g. Ellerby and Askew, 2007). Muscle activity is also regulated at the molecular level in ways that affect both mechanical performance and energy consumption rate (Tian et al., 2001; Narolska et al., 2005; Hoyer et al., 2007). Together, these mechanisms allow muscle to meet mechanical demands while adhering to constraints on energy supply rate. Mammalian heart muscle has received most attention for the functional effect of molecular variation, owing to the heart's mechanical need to circulate blood throughout the body and to obtain oxygen *via* its own pumping. This requires a precise match between mechanical performance and the supply and consumption rate of oxygen, with fatal consequences for even a temporary mismatch. Flight muscles may have a similar need for molecular-level fine tuning, as flying animals can have a severely reduced ability to function on the ground and therefore their flight muscles must be able to support the body weight while working within the constraints imposed by energy supply. In the study presented here, we tested this hypothesis by determining whether insect flight muscles make adjustments at the molecular level in response to variation in body weight and nutrition, and how that variation is related to flight energetics and life history.

The thin filament regulatory complex (troponin–tropomyosin) switches muscle contraction on and off in response to changes in the intracellular concentration of calcium (Gordon et al., 2000). When muscle is more sensitive to calcium, more cross-bridges are activated during a contraction and more force is generated, at the cost of greater ATP consumption (Greaser et al., 1988). Hence, variation in the molecular composition of the regulatory complex is predicted to affect both muscle performance and energy consumption rate. In this study, we focused on one gene in the thin filament regulatory complex, *troponin-t* (*Tnt*). In all animal species examined to date, *Tnt* undergoes alternative splicing (AS) to form multiple protein isoforms that differ in their amino acid sequence and function. Experiments with vertebrate striated muscle have consistently shown that manipulation of *Tnt* isoform content affects muscle calcium sensitivity and contractile performance (Ogut et al., 1999; Gomes et al., 2002; MacFarland et al., 2002; Gomes et al., 2004; Nassar et al., 2005; Brotto et al., 2006; Chandra et al., 2006). Single amino acid replacement mutations in human *Tnt* have similar effects (Hernandez et al., 2005). Functional differences in muscle activation rate along the body axis of rainbow trout correlates with changes in *Tnt* isoform profiles (Coughlin et al., 2005). In the flight muscles of dragonflies, increases in the relative abundance of larger *Tnt* transcripts are associated with increases in calcium sensitivity, force, power and the energetic cost of flight (Marden et al., 1999; Marden et al., 2001). Variation in dragonfly flight muscle power output is in turn related to the territorial and mating success of individual males (Marden and Cobb, 2004),

thereby demonstrating the link between muscle performance and fitness.

Although these studies reveal strong effects of *Tnt* variability, there is little understanding of what causes variation in isoform composition within muscles of different individuals. More generally, even though approximately 50–75% of animal genes undergo AS at the pre-mRNA level (Maniatis and Tasic, 2002), there is fairly rudimentary knowledge regarding the control of AS by extracellular signals (Lynch, 2007). AS is well known for providing tissue-specific function and performing key roles in development, but there has been little effort to examine how quantitative intraspecific variation in AS within a tissue and developmental stage affects phenotype and fitness (Marden, 2008). Our study directly examined the relationship between whole-organism variables, AS and components of fitness. We tested the hypothesis that larger *Tnt* isoforms increase in relative abundance as body weight increases, as nutritional condition improves, and are associated with higher flight metabolic rate. We also tested the related hypothesis that the *Tnt* isoform profile is a marker of organismal condition by examining its association with the behavior and fecundity of free-living insects.

MATERIALS AND METHODS

We used two insect species, the fall armyworm moth [*Spodoptera frugiperda* (J. E. Smith); Lepidoptera; Noctuidae] and the Glanville fritillary butterfly (*Melitaea cinxia* L.; Lepidoptera; Nymphalidae) to test our hypotheses. Armyworm moths were reared in the lab on an artificial diet, with experimental manipulations of larval feeding that affected adult size and nutritional condition. Adult fritillary butterflies were captured from the wild and observed in a large outdoor population cage in order to determine how the *Tnt* isoform profile relates to the performance and fitness of free-living insects following larval development on wild plants.

Moths

Eggs of the fall armyworm rice strain were obtained from C. Dillard and R. Meagher (USDA ARS Center for Medical, Agricultural and Veterinary Entomology, Gainesville, FL, USA). Larvae were reared individually on cubes of a pinto bean artificial diet. All stages were reared at 27°C, 70% relative humidity, and 14 h:10 h (L:D) photoperiod. Fourth instar larvae with slipped head capsules and weighing greater than 160 mg were selected in order to obtain developmentally synchronous larvae that began the last instar at a similar size (Fescemyer et al., 1986). After eclosion, virgin adult moths were housed separately by gender and fed *ad libitum* a solution containing 6% honey and 0.2% ascorbic acid.

Butterflies

In early June 2004, 176 adult *M. cinxia* with fresh and undamaged wings from 25 populations around the Åland Islands were caught, weighed and individually marked underneath their hind wing. On the evening of the capture, butterflies were released into a large outdoor population cage (32 m×26 m×3 m), in which the life history experiment was conducted (Hanski et al., 2006; Saastamoinen, 2007a; Saastamoinen, 2007b; Saastamoinen, 2008). A subset of females that survived to the end of the experiment (N=21 from 16 populations) was sampled for *Tnt* isoform composition. The number of days that butterflies were observed in the cage varied from 12 to 20 (mean=17, s.d.=2).

Manipulation of larval diet and adult weight

Larval nutrition of armyworm moths was manipulated using variations within three experimental regimes: (1) starving last instars for the first 1–4 days before placing them back on diet to complete larval development, (2) allowing larvae to feed on diet for the first 1–3 days before allowing them to complete larval development while being starved, and (3) allowing last instars to feed on diet the whole time. During starvation, larvae were fed an indigestible gel (Gelcarin®, a refined carrageenen used as a food-grade gelling agent; FMC, Food Ingredients Division, Rockland, ME, USA) to provide digestive bulk and water.

In a second experiment, adult weight was manipulated independently of larval feeding history by gluing a small piece of lead (i.e. 50–80 mg; 42–82% of unladen body mass) to the dorsal abdomen of adults within the first 6–8 h after eclosion. Four treatment groups were formed using combinations of restricted (fed 2 days then starved) *versus* unrestricted larval diet and presence *versus* absence of body weight manipulation. Weight loads were attached on day 1 of adult life and remained attached until sampling on adult day 5. All moths were maintained in cages as described above, where they flew frequently, as evidenced by wing wear and scale loss. This indicates that the flight muscles had a number of days of experience working against the manipulated body weight.

Cloning and characterization of *Tnt*

Conserved regions at the 5′ and 3′ ends of the coding region of known insect *Tnt* genes were used to design degenerate primers to amplify a 1.1 kb fragment of *Tnt*, which we then cloned and sequenced. These initial primers were SfTNTF-5841 (5′-GGAGCAGCTGGAGGAGGARAARAARAT-3′) and SfTNTR-5845 (5′-CCTTGTGCCGCAGCTGYTGYTTYTG-3′) for the armyworm, and McTNT-1 (5′-CATGTCBGACGAKGARGARTA-3′) and McTNT-4 (5′-TTCACCAARCCRCTCGCTGG-3′) for the fritillary. The 5′ and 3′ ends of the mRNA were obtained by RACE (BD Biosciences Clontech, Palo Alto, CA, USA) using gene-specific primers. These RACE primers were SfGSP1-8617 (5′-AGTCCTGCCTCTTTTGCCTCTCCTC-3′) and SfGSP2-8616 (5′-GAAAATCTCGCTGTCCATCCGCATC-3′) for the armyworm, and McTNT-7 (5′-CGCTTCTCTTCCTCAGCGCGAGATAC-3′) and McTNT-8 (5′-AGGCACAAGGCCCTCAAGAAAGGTCT-3′) for the fritillaries. Sequences identified with 5′-RACE included 127 nucleotides of the 5′-UTR. The alternatively spliced region of *Tnt* begins 23 bases after the start codon, and therefore the 5′-UTR sequence enabled us to design fluorescently labeled forward gene-specific primers lying in the 5′-UTR just outside the 5′-end of the coding region (SfTntAltF 5′-56FAM-CACCCGTGCGAC-ATTAAATAAAC-3′, McTnTF 5′-56FAM-AACCCGTGCGAC-ACTAATAAATC-3′). These were paired with reverse gene-specific primers (SfTntAltR 5′-GCGCCATTCGTTGATGTATTC-3′, McTnTR 5′-GACTACATCAACGAATGGCGTA-3′) to yield gene fragments containing constitutively spliced regions on both sides of the 5′ alternatively spliced region.

Tnt isoform profiling

Flash-frozen moth or butterfly thoraces were placed in a tube containing a 5 mm steel bead and frozen in liquid nitrogen. TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) was subsequently added to this tube and the tissue disrupted and homogenized on a mixer mill. Insoluble material was removed by centrifugation, and total RNA was isolated from the homogenate supernatant by the TRIzol® manufacturer's protocol, except for an additional acid phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v) phase separation step prior to RNA precipitation to reduce DNA contamination. A preliminary experiment found this extraction method to yield high

quality total RNA with no detectable DNA contamination and RNA integrity numbers ranging from 7.7 to 9.8.

The RNA was quantified with a UV spectrophotometer and an aliquot providing 0.5 μg of RNA was used in a cDNA synthesis with Powerscript (BD Biosciences Clontech) or Superscript II (Invitrogen) using oligo(dT)$_{18}$. The spliced region of *Tnt* was amplified from 1 μl of this cDNA using standard PCR for 25 cycles with GoTaq DNA polymerase and the 56FAM fluorescently labeled primers described above. Capillary electrophoresis of the resulting labeled *Tnt* fragments was performed on an ABI Hitachi 3730XL DNA Analyzer (Foster City, CA, USA). Internal size standards (GeneScan™-500LIZ, Applied Biosystems, Foster City, CA, USA) and GeneMapper® (Applied Biosystems) fragment analysis software were used for determination of peak size and height. Before capillary electrophoresis, products in the PCR were diluted (usually 1:25) in water so that all isoform peak heights fell within the linear range (i.e. below 30,000 units) of the instrument detector.

All data reported for *Tnt* isoforms are based on relative peak height values calculated by dividing the peak height for a particular isoform from an individual insect by the sum of the peak heights for all isoforms detected in that insect's thorax. Results were essentially identical if we used the area under peaks rather than peak height. Preliminary experiments found relative peak height values were only trivially influenced by DNase treatment of the RNA and variations in PCR reaction components (0.5–2.5 mmol l^{-1} magnesium ion, 0.1–1.0 μmol l^{-1} primer, 20–200 μmol l^{-1} dNTP), annealing temperatures (54–63°C) or cycle number (20–35).

To determine whether these alternative transcripts were translated to protein, we performed a western blot using homogenized armyworm flight muscle protein and MAC145 (Bullard et al., 1988), a monoclonal antibody that hybridizes with insect Tnt protein. Individuals used for protein blotting were also assayed for relative abundance of *Tnt* transcripts so that we could assess the relationship between isoform transcript and protein abundance.

Flight metabolic rate

Moths from the larval nutrition treatments were placed inside a transparent 1 l cylindrical jar through which dry CO_2-free air was flowed at 0.95 l min^{-1}. These moths were stimulated to fly in a nearly continuous fashion by tapping the jar whenever they alighted. Air temperature ranged from 23 to 25°C. After a steady 2–3 min baseline of resting CO_2 emission before flight had been established, moths were stimulated to fly for 10 min. Respirometry experiments were performed blindly with regard to larval nutrition treatment. A calibrated LiCor 6252 gas analyzer (Lincoln, NE, USA) was used to determine CO_2 concentration. Flow rate control and AD conversion were accomplished using Sable Systems instruments (Las Vegas, NV, USA). We subtracted the mean pre-flight CO_2 emission rate (resting metabolism) and used standard equations for open-flow respirometry (Lighton, 1991) and a *Z*-transformation to remove time lags (Bartholomew et al., 1981) to determine the peak rate of CO_2 emission attributable to flight metabolism. Adults were flown at 3–7 days after eclosion.

We found in a separate experiment (data not shown) that moths challenged with acutely attached weight loads did not increase their peak flight metabolic rate. In this experiment, peak flight metabolic rate depended on thorax mass (i.e. flight muscle) rather than total weight (body plus attached load), which suggests that our measured peak rates in general reflect the maximum performance of the flight muscles. For this reason we used thorax mass rather than body weight as the independent variable representing size in our analyses of flight metabolic rates.

Butterfly life history

The population cage (32 m×26 m×3 m), located outdoors in a meadow in Åland, Finland, was divided into 8×8 grid cells that were systematically surveyed every second hour between 09:00 and 17:00 h. During surveys the location and activity of each butterfly observed were recorded. Butterflies were categorized as basking (wings open), resting (wings closed) or flying. Females were provided with potted host plants (*P. lanceolata* and *V. spicata*) in the central part of the cage, which was relatively bare of other vegetation. The host plants were continuously monitored to record individual ovipositions. After a female completed oviposition, her eggs were removed and counted at the age of 3 days. As all butterflies were marked, we were able to gain information about the individual rate of egg production. Hatch percentage of eggs was high (>90%) in nearly all cases, so we used the total egg count because it involved less handling of the delicate eggs and may therefore reduce error. Naturally occurring flowering plants within the cage were abundant and provided nectar for the adults. Butterflies were sampled and preserved in liquid nitrogen either during the experiment if they were no longer able to fly (i.e. wings were too worn) or lay eggs, or at the end of the experiment, during 21–24 June.

RESULTS

Lepidopteran *Tnt*, like all *Tnt* genes examined to date, contains a series of alternative exons near the 5′ end that are variably incorporated into mRNA (Fig. 1). Exon identity was confirmed by alignment of isoforms against *Bombyx mori* genomic and cDNA *Tnt* sequences. Consistent alignment of the isoforms of the three species, along with unambiguous identification of homologous conserved exons within the *B. mori* genomic sequence, showed that exon structure and alternative splicing of this gene are highly conserved in Lepidoptera.

Two pairs of isoforms (A/B and C/D) differed only by the presence/absence of a single 3-nucleotide microexon, and these pairs together with *Tnt F* comprised the most abundant transcripts (Fig. 1). Relative abundances of the different *Tnt* isoforms varied widely between individuals, at both the transcript and protein level (Fig. 1). Thus, *Tnt* isoform transcripts are translated to proteins that have similar relative abundances.

To test the hypothesis that relative abundances of the *Tnt* transcripts respond to nutrition, we experimentally varied the number of days that fall armyworm larvae had access to artificial diet. This treatment had strong effects on the duration of the final larval instar and on adult body mass (Fig. 2). In 3–7 day old adults, the relative abundance of the smallest and most common isoform (*Tnt F*) decreased with increasing larval access to food (Fig. 3A; $P<0.0001$), while the relative abundances of larger isoforms increased. Larval nutrition affected body size, and hence there was a tight correlation between *Tnt* isoform composition and body mass ($R^2=0.82$, $P<0.0001$; Fig. 3B). Flight muscles must counteract gravitational force on the body, and therefore it is possible that *Tnt* splicing responded to body weight rather than mass or nutrition *per se*. There was, however, a significant weight-independent effect of larval nutrition on the relative abundance of *Tnt F* (Fig. 3C; $P=0.02$), which indicates that feeding history during the larval stage affected the molecular composition of muscle in adults even when they reached the same size.

To better distinguish the independent effects of nutrition and body weight, we performed a second experiment in which these variables were decoupled. Four treatment groups were formed using combinations of restricted (fed 2 days then starved) *versus* unrestricted larval diet and presence *versus* absence of body weight

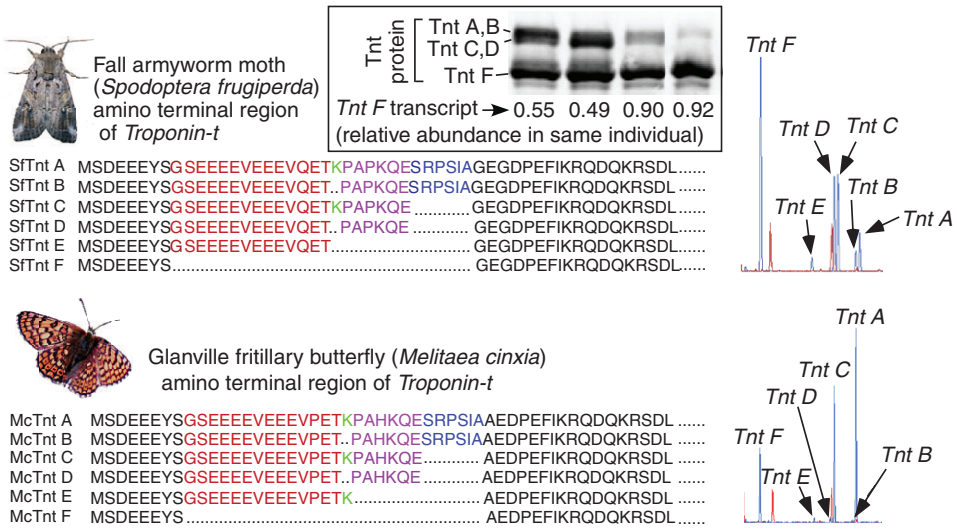

Fig. 1. Variable N-terminal region of protein isoforms produced by alternative splicing of the *Tnt* gene in fall armyworm moths and Glanville fritillary butterflies. Alternative exons are colored. Traces on the right show capillary electrophoresis separation of PCR products generated by primers that hybridize on either side of the alternative exon region and from which we determined fragment size and relative abundance. Fragment size measurements match predicted sizes from sequence data. Red peaks are internal size standards. Inset shows western blot of moth flight muscle protein separated by SDS-PAGE and hybridized with MAC 145 antibody specific to insect Tnt. Relative abundances of *Tnt F* transcripts from the same individuals (below each lane) show agreement with the Tnt F protein isoforms. Specifically, these data show that Tnt F was the predominant protein form when *Tnt F* transcript relative abundance was high, and was about half of the protein when *Tnt F* was about 50% of the transcript pool. (Adult moth photo courtesy of Renn Tumlison.)

manipulation (small lead weights attached to the abdomen of the adult moth). Loads were attached on day 1 of adult life and moths were sampled at day 5. The relative abundance of the predominant isoform, *Tnt F*, varied significantly with total weight (body+attached load) and nutrition treatment (Fig. 4; Table 1), with no effect of gender. This result confirms that AS of *Tnt* responds to both weight and nutrition. Strikingly, the effect of experimentally added weight on *Tnt* splicing was nearly identical to the corresponding effect of increased body tissue mass, and there was no relationship between the actual body weight (without the load) and the relative abundance of *Tnt F* (Fig. 5).

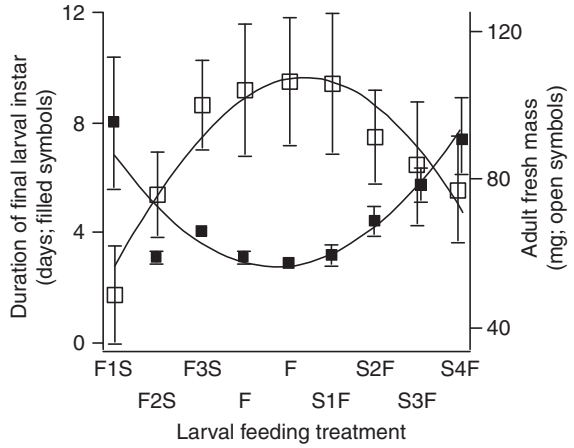

Fig. 2. Duration of the final larval instar and adult fresh body mass for moths reared in different feeding treatments (N=108). Labels indicate the number of days that larvae were fed prior to removal of food (i.e. F1S were fed 1 day, then starved until pupation) or the number of days starved prior to feeding until pupation (i.e. S4F were starved 4 days, then fed until pupation). Two groups were fed the whole time (F).

Relative abundances are necessarily correlated, but it appears that individual isoforms responded dissimilarly to the different treatment variables. Four isoforms were associated with total weight (*Tnt F*, *Tnt C*, *Tnt B*, *Tnt A*; Fig. 4; Table 1) and one with gender (*Tnt E*), while the remaining one (*Tnt D*) showed a complex pattern, being associated with weight when total load was less than 100 mg but with nutrition when the load exceeded 100 mg (Fig. 4).

Moths with better feeding history and greater body mass had proportionately larger abdomens, causing their flight muscles to be more heavily loaded during flight (ratio of abdomen/thorax mass increased by 50% from the smallest to the largest moths; P<0.0001). To examine how *Tnt* isoforms relate to energy consumption, we used moths (N=88) from the first nutrition experiment (no weight attached) and measured their peak rate of CO_2 production during flight (Fig. 3D), which in insects is dominated by flight muscle metabolism. Thorax mass (i.e. flight muscle mass; P=0.0013), gender (P<0.0001) and relative abundance of *Tnt F* (P=0.004) had highly significant effects [larval feeding treatment had no independent effect (P=0.8) and was dropped from the model]. Thus, *Tnt* isoform composition was associated with the muscle size-independent energy consumption rate, which is presumably important when the load on the flight motor increases due to better nutritional history and a heavier body.

We next tested the hypothesis that alternative splicing of *Tnt* in flight muscles correlates with other phenotypically plastic traits that may together adjust function in relation to body size and nutrition. We accomplished this by comparing *Tnt* isoform composition with life history and fecundity in fritillary butterflies. Newly eclosed butterflies had *Tnt* isoform profiles similar to those of 3–7 day old adult moths (data not shown), but the profile shifted to higher relative abundances of the larger isoforms, particularly *Tnt A* (Fig. 1), later in life. The relative abundance of *Tnt A* at the end of adult life was

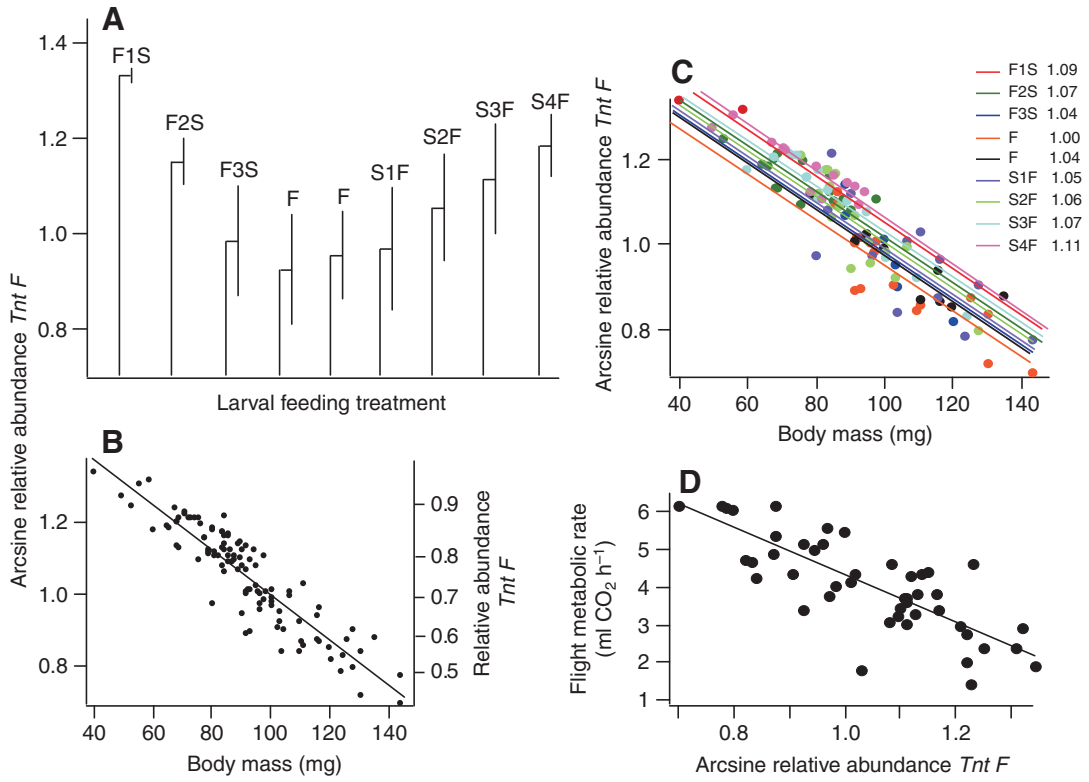

Fig. 3. *Tnt F* (means ± s.d.) in flight muscles of groups of moths (*N*=108) representing different larval feeding treatments (A) and in relation to body mass (B,C). Ratios are arcsine transformed to achieve normality; right axis in B shows the scale of untransformed relative abundances. Labels in A indicate the number of days fed during the final larval stage prior to removal of food or the number of days starved prior to feeding until pupation (as in Fig. 2). C shows regression lines and the least squares means from a multivariate model with body mass and feeding treatment (no significant interaction). (D) Peak flight metabolic rate in relation to relative abundance of *Tnt F* in female moths.

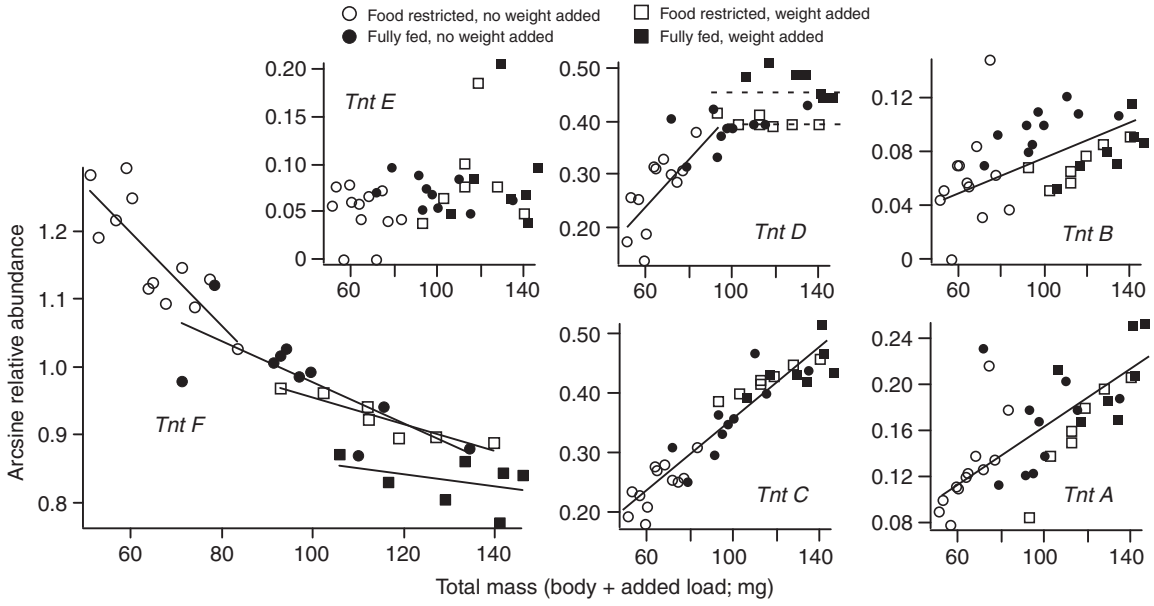

Fig. 4. Relative abundances of the six isoforms of *Tnt* in the fall armyworm moth in relation to total mass (body+added load) and larval feeding treatment (fully fed *versus* fed 2 days then starved). Lines in the large plot show individual regressions for each of the four treatment groups for the most abundant isoform, *Tnt F*. Smaller plots show effects of total mass (solid lines) or nutrition (dashed lines for *Tnt D* at total mass >100 mg) in the remaining five isoforms.

Table 1. The *P*-values and proportion of variation explained (r^2) for the relative abundance of six isoforms of *troponin-t* in fall armyworm flight muscles from an experiment that combined larval feeding treatment with a weight-loading treatment

Isoform	Total weight	Larval feeding treatment	Load treatment	Gender	r^2
Tnt F	**<0.0001**	0.01	0.04	0.52	0.89
Tnt E	0.59	0.71	0.40	**0.002**	0.39
Tnt D	0.04	**0.005**	0.04	0.26	0.76
Tnt C	**<0.0001**	0.76	0.26	0.37	0.89
Tnt B	**0.0005**	0.23	**0.005**	0.49	0.60
Tnt A	**0.004**	0.23	0.73	0.89	0.61

Body weight (unladen mass plus added load) was treated as a continuous variable and the presence or absence of an experimental load (load treatment) was treated as an additional categorical variable. *P*-values that are significant at an experimentwise type 1 error rate of 0.05 (Bonferroni adjusted critical *P*-value=0.0080) are shown in bold. *N*=35 moths.

positively correlated with activity (times per day observed basking, R^2=0.26, P=0.02; Fig. 6A), the rate of egg production (R^2=0.60, P<0.0001; Fig. 6B), and total egg production while in the cage (R^2=0.62; P<0.0001). Egg quality, as judged by the percentage of eggs that hatched, did not vary with *Tnt A*, daily egg production rate or total eggs produced (P>0.3 in each case).

DISCUSSION

These results support the hypothesis that insect flight muscles make adjustments at the molecular level in response to variation in body weight and nutrition, and that this variation is related to flight energetics and life history. Restricted access to food during the larval stage affected adult body mass and the *Tnt* isoform composition in flight muscles. The *Tnt* isoform profile could in theory have responded to either nutritional effects or gravitational loads on the muscles created by the body weight. Strong support for the latter was provided by the striking response of all moths to chronic addition of external weight (Figs 4 and 5). In addition to the major effect of weight, there was also a subtle effect of nutrition. Independent of body weight, moths from more restricted larval feeding regimes produced more of the small *Tnt* isoforms (Fig. 3C; Table 1) that are associated in other insects with weaker muscles (Marden et al., 2001) and in the present experiment with reduced energy expenditure rate. Similarly, moths that experienced greater food restriction as larvae responded less to external weight (Fig. 4A). These results imply the existence of distinct pathways for signals carrying information about body weight and nutrition. We suggest that the purpose of this plasticity in *Tnt* isoform expression is to adjust muscle force output according to gravitational load, and to make additional finer scale adjustments to energy consumption rate in order to accommodate variation in nutritional state.

The most severe food restriction resulted in delayed pupation (Fig. 2), low survival (12%) and very small adult size, down to 37% of the average mass of moths that developed from fully fed larvae. Minimal body size corresponded with the relative abundance of *Tnt F*

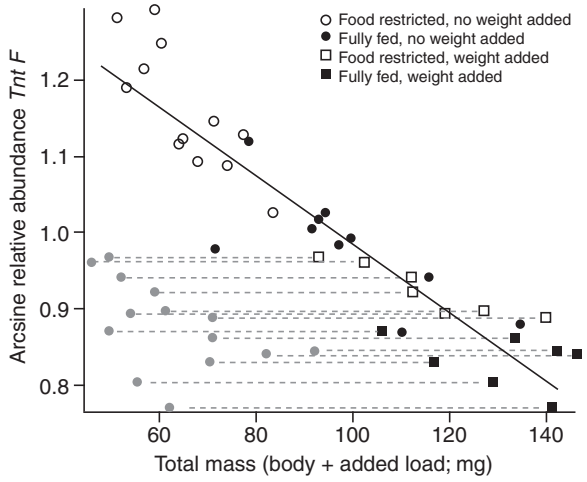

Fig. 5. Relative abundance of *Tnt F* in fall armyworm moths as a function of total mass (body+added load). Superimposed on the plot are gray points showing the unladen body mass of the weight-manipulated individuals, with horizontal lines connecting their unladen mass to their total mass after attachment of a weight load. The vertical distance from the fitted line to the gray points is the difference between the observed and predicted *Tnt F* relative abundance if there had been no adjustment of *Tnt F* to the added weight. The reduced unladen body mass of the fully fed weight-loaded moths was presumably caused by the increased cost of activity over 5 days while weight loaded or by active reduction in body mass, or both.

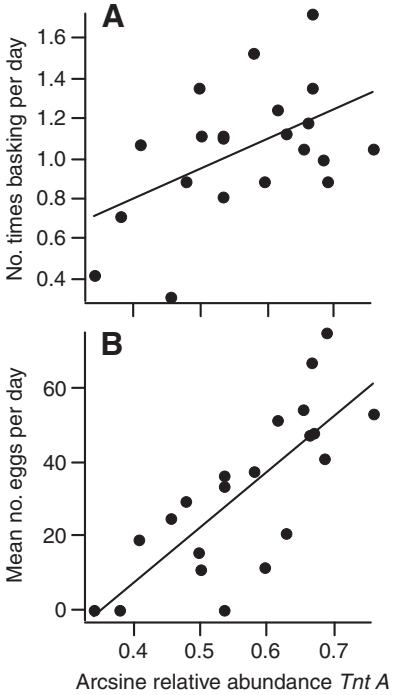

Fig. 6. Relative abundance of *Tnt A* in the flight muscles of Glanville fritillary female butterflies in relation to (A) the number of times per day butterflies were observed perching with open wings (basking; thermoregulation in preparation for flight) and (B) the rate of reproduction (number of eggs laid per day). The relationships in A and B both show statistical significance that is independent of initial body mass.

reaching 95% (Fig. 3B), and therefore it appears that limits to viable body size and to the range of the *Tnt* isoform adjustability (i.e. near 100% representation of a single isoform) are coupled. Correspondence between the range of viable body sizes and size-dependent molecular level variation may be an evolutionary outcome rather than a coincidence. This hypothesis could be tested in future work by comparing the range of isoform relative abundance with intraspecific variation in body size among species that differ widely in size, with the prediction being that the *Tnt* isoform response is consistently saturated at extremes of size variation. The *Tnt* isoform response may also contribute mechanistically to the isometric scaling of muscle force within and between species (Marden, 1987; Dillon and Duley, 2004), as it is clear from the present result that isoforms are adjusted in response to changes in body weight. Previous work in insects has shown that *Tnt* isoforms are associated with muscle force production (Marden et al., 2001), and so the plasticity of *Tnt* isoform expression may be among the mechanisms that allow animals to produce sufficient force, which depends on muscle cross-sectional area, as volume-dependent body weight increases (Schilder and Marden, 2004).

Moths with greater body weight, better nutritional condition and more of the large *Tnt* isoforms showed higher flight metabolic rate in relation to thorax mass and gender (Fig. 2D). We do not yet know if this provides a motility advantage for these individuals, but Glanville fritillary butterflies with higher flight metabolic rate fly greater distances in nature (K. Niitepõld, A. D. Smith, J. L. Osborne, D. R. Reynolds, N. L. Carreck, A. P. Martin, J.H.M., O. Ovaskainen and I.H., manuscript in preparation), and we have found in field studies of other insects (dragonflies) that males possessing higher muscle power output were better able to defend territories and compete for mates (Marden and Cobb, 2004). Fall armyworm moths are nocturnal and migrate over long distances, so we could not observe them in the field to test hypotheses about the effects of different muscle traits on their performance. Butterflies are much better models for adult behavior, and by observing Glanville fritillary butterflies we found that females with more of the large *Tnt* isoforms basked more often (i.e. more sun-seeking thermoregulatory behavior in preparation for flight) and produced more eggs (Fig. 6). Thus, relationships between isoform profiles and organismal quality that we observed for moths reared in the lab on artificial diet were also apparent in the life history and fitness of butterflies that developed on wild plants and were kept as adults in a large outdoor population cage. Mechanisms underlying the correlation between the *Tnt* isoform profile and fecundity are uncertain, but it is worth noting that egg production in other insects is regulated by ovarian genes that are alternatively spliced according to nutritional condition (Terashima and Bownes, 2004) and which might be co-regulated with AS pathways in muscle.

The discovery that muscle performance is adjusted by evolutionarily conserved AS in response to weight and nutrition opens the door for an examination of the pathways by which this occurs and the impact on energy budgets and mobility. Previous work on muscle plasticity suggests that both cell-autonomous and systemic mechanisms may be involved. For example, mechanical stress affects AS of the spring-like *titin* gene (Granzier et al., 2007), indicating that striated muscle may contain a cell-autonomous weight sensor. Mechanical stress also affects alternative splicing of the *insulin-like growth factor-1* gene, to form a mechano-sensitive isoform (McKoy et al., 1999) that stimulates muscle stem cell proliferation (Hill and Goldspink, 2003). Additional evidence for systemic factors comes from the observation that AS of the vertebrate *insulin receptor* gene, a key component of nutrient signaling, is co-regulated (Ho et al., 2004) with AS of *Tnt*. These results indicate that there may be a mix of systemic and intracellular signals and pathways that influence AS and the molecular composition of muscle contractile proteins.

The tight association between *Tnt* isoform plasticity, larval feeding and adult size indicates that this molecular adjustment is one of the ways that organisms maintain functional homeostasis despite unpredictable adult body size. Progression to the pupal stage in insects is determined in large part by attainment of a critical size during the final larval instar (Nijhout et al., 2006), and therefore adult insects are much less variable in size than animals that have indeterminant growth. However, our experiment that varied food availability during the final larval instar led to adult moths of widely divergent body mass (range 40–149 mg), and this must to some extent reflect the way natural variation in foliage quality and weather affects larval feeding and growth (Davidowitz et al., 2003). Thus, we suggest that the ability to adjust *Tnt* isoform composition, with consequent changes in muscle performance and energy consumption rate, has adaptive value for coping with the variation in adult body size and weight that arises from unpredictable differences in larval feeding. This type of molecular adjustment may also be a mechanism underlying the long-lasting changes in performance and life history (Metcalfe and Monaghan, 2001; Metcalfe and Monaghan, 2003) that have been observed in a variety of animals after food restriction in early life, reduced early growth rate and subsequent compensatory growth. We found evidence for such an effect in the adult mass-independent effect of larval food restriction on *Tnt* isoform composition of moths (Fig. 3C).

AS has been viewed predominantly as a mechanism for generating tissue-specific and developmental stage-specific function required to build a complex organism (Maniatis and Tasic, 2002). In addition to these roles, our results show that within a stage and a tissue, AS responds to organismal condition to produce different isoform profiles that are associated with energetics and life-history traits. Hence, AS functions not only as a switch but also as a dial, in this case to control the amplitude of muscle force output and energy consumption rate. Genes with known AS have not been widely examined in this fashion, but there are hints in the literature that AS may commonly have such a role in continuously adjusting organismal traits (Marden, 2008). On the other hand, some genes with AS show the other extreme, a remarkably invariant isoform profile among individuals (Chisa and Burke, 2006). Apparently, there is broad variation in the way animals use AS. Our discovery that AS can be quantitative and strongly associated with key features of organismal condition, performance and fitness provides an impetus for further exploration of this aspect of phenotypic plasticity and homeostasis.

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