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## Parasites, proteomics and performance: effects of gregarine gut parasites on dragonfly flight muscle composition and function

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### Summary

In previous work, we found that dragonflies infected with gregarine gut parasites have reduced muscle power output, loss of lipid oxidation in their flight muscles, and a suite of symptoms similar to mammalian metabolic syndrome. Here, we test the hypothesis that changes in muscle protein composition underlie the observed changes in contractile performance. We found that gregarine infection was associated with a 10-fold average reduction in abundance of a ~155 kDa fragment of muscle myosin heavy chain (MHC; ~206 kDa intact size). Insect MHC gene sequences contain evolutionarily conserved amino acid motifs predicted for calpain cleavage, and we found that calpain digestion of purified dragonfly MHC produced a peptide of ~155 kDa. Thus, gut parasites in dragonflies are associated with what appears to be a reduction in proteolytic degradation of MHC. MHC155 abundance showed a strong negative relationship to muscle power output in healthy dragonflies but either no relationship or a weakly positive relationship in infected dragonflies. Troponin T (TnT) protein isoform profiles were not significantly different between healthy and infected

dragonflies but whereas TnT isoform profile was correlated with power output in healthy dragonflies, there was no such correlation in infected dragonflies. Multivariate analyses of power output based on MHC155 abundance and a principal component of TnT protein isoform abundances explained 98% of the variation in muscle power output in healthy dragonflies but only 29% when data from healthy and infected dragonflies were pooled. These results indicate that important, yet largely unexplored, functional relationships exist between (pathways regulating) myofibrillar protein expression and (post-translational) protein processing. Moreover, infection by protozoan parasites of the midgut is associated with changes in muscle protein composition (i.e. across body compartments) that, either alone or in combination with other unmeasured changes, alter muscle contractile performance.

Key words: dragonfly, infection, muscle performance, muscle protein composition, myosin heavy chain, troponin T.

### Introduction

Dragonfly flight muscles are convenient models for studying the mechanics and plasticity of muscle contractile performance (Marden et al., 2001; Schilder and Marden, 2004) and how this variation affects the outcome of aerial competition in nature (Marden and Cobb, 2004). Recently, we have found that infection of the dragonfly midgut by gregarine parasites (Protozoa: Apicomplexa) is an important contributor to variability in muscle performance. Gregarine infection causes decreased male territorial and mating success in odonates (Plaistow and Siva-Jothy, 1996; Marden and Cobb, 2004) and causes impairment of *in vitro* muscle lipid metabolism and power output (Schilder and Marden, 2006). Here, we explore molecular mechanisms underlying some of these phenotypes by examining how gregarine infection affects flight muscle protein composition and its relation to flight muscle contractile performance.

Previous mechanistic examinations of parasite effects on muscle have been restricted to specialized parasites such as

*Trichinella spiralis* that directly infect muscle cells (Jasmer, 1990; Jasmer and Kwak, 2006), causing local pathology. Here, we take the different approach of examining a systemic effect of non-invasive parasites residing in the gut. Our results show that dragonfly skeletal muscle is sensitive and responsive to the presence of parasites in other tissues, with specific effects on muscle protein composition.

Effects of parasites on muscle performance are best examined in the context of the dynamic and versatile nature of healthy skeletal muscle. Skeletal muscle consists of cytoskeletal networks (Clark et al., 2002) that provide the foundation for contractile activity. Structural integrity and quality of cytoskeletal networks are maintained through continuous protein turnover – a balance between protein degradation and synthesis (Rooyackers and Nair, 1997; Mykles, 1998). In addition, contractility depends on the types of myofibrillar proteins expressed in sarcomeres (i.e. protein isoform composition). Both protein turnover and isoform composition

are sensitive to changes in nutritional status and exercise intensity (White et al., 2000; Ferrando et al., 2002; Wackerhage and Rennie, 2006) and are affected by aging and disease (e.g. Short and Nair, 2001; Nair, 2005; Du et al., 2004). Proteomic studies of muscle contractility to date have focused exclusively on protein composition but not at all on proteolytic degradation of proteins, the latter of which is a likely first-order indicator of cytoskeletal integrity and/or turnover. In the present study, we examine metrics of both protein degradation and isoform composition that, for healthy dragonfly flight muscle, provide strong explanatory power for variability in contractile performance.

In vertebrates, fibers containing different myosin heavy chain (MHC) protein isoforms have different metabolic and mechanical characteristics, and their relative abundance in a muscle fiber determines speed of contraction and metabolic profile [i.e. fiber type designation; slow or fast, glycolytic or oxidative (Pette and Staron, 2001)]. Shortening velocity and  $Mg^{2+}$ -ATPase activities vary with MHC protein isoform composition (Bottinelli et al., 1991; Bottinelli et al., 1994) and, for many muscles, fiber type distributions change in response to exercise, with age and during disease.

In the best-studied insect, *Drosophila*, alternative mRNA splicing of a single MHC gene gives rise to at least 14 mRNA transcripts (Vigoreaux, 2001). However, only one MHC protein isoform is expressed in *Drosophila* flight muscles (Bernstein et al., 1986) and little is known about MHC isoform composition of flight muscles of any other insects; this raises the question of how insects regulate and vary muscle performance.

One mechanism that insects use to accomplish plasticity of muscle contractile performance is alternative splicing of mRNA coding for the myofibrillar protein troponin T (TnT). Alternative splicing of TnT in *Libellula pulchella* dragonflies (Odonata: Anisoptera) produces seven mRNA transcripts, six of which are found in flight muscles. The relative abundance of particular TnT mRNA transcripts correlates positively with muscle fiber  $Ca^{2+}$  sensitivity, *in vitro* muscle performance and kinematic measures of *in vivo* flight performance (Marden et al., 1999; Marden et al., 2001), but the relationship between TnT splice form variation and contractility has not yet been examined quantitatively at the protein level.

In vertebrates, variation in TnT isoform composition of muscle fiber types corresponds tightly with variation in MHC isoform identity, indicating that MHC expression and TnT alternative splicing are co-regulated (Galler et al., 1997). MHC and TnT expression profiles have not been examined together in studies of insect muscle contractile performance. Here, we use two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and MALDI-TOF mass spectrometry to quantify expression of TnT isoforms and the abundance of an identified product of MHC proteolytic degradation in dragonfly flight muscle. We compare these metrics in healthy and infected dragonflies and their relationship to *in vitro* flight muscle performance.

## Materials and methods

### Animals

Mature adult male *Libellula pulchella* dragonflies were netted at two ponds in the State College, PA, USA area. After capture, dragonflies were transported to the laboratory in

cooled plastic containers containing moist paper. Infection status was assessed by surgically opening the midgut after decapitation (for details, see Marden and Cobb, 2004). Directly after flight muscle performance measures (see below), muscle tissues were stored in liquid nitrogen until further analyses were performed. For none of the parameters examined here did we observe a dosage effect based on the number of gregarines present in the midgut, which is why comparisons are between healthy and infected groups.

### 2-D PAGE

For electrophoretic analyses, we chose a subset of basal muscle samples that spanned the variation observed in a previous study in which we measured muscle power output and gregarine infection status (Schilder and Marden, 2006). A total of 17 muscle protein samples was prepared and separated using 2-D PAGE. Sample preparation was performed according to instructions provided with a MK-1 tissue preparation kit (Kendrick Labs, Madison, WI, USA). Basal muscles tested for contractile performance were dissected out and homogenized in osmotic lysis buffer [10 mmol  $l^{-1}$  Tris, pH 7.4, 0.3% sodium dodecyl sulfate (SDS)] containing 1% protease inhibitor cocktail [20 mmol  $l^{-1}$  4-(2-aminoethyl) benzenesulfonyl fluoride, 1 mg  $ml^{-1}$  leupeptin, 0.36 mg  $ml^{-1}$  E-64, 500 mmol  $l^{-1}$  EDTA and 5.6 mg  $ml^{-1}$  benzamide]. Homogenates were heated at 95°C in SDS boiling buffer (5% SDS, 5%  $\beta$ -mercaptoethanol, 10% glycerol and 60 mmol  $l^{-1}$  Tris, pH 6.8). Samples were then shipped to Kendrick Labs for 2-D PAGE separation. Isoelectric focusing (IEF) was performed in a glass tube (inner diameter 2 mm) using 2.0% pH 3.5–10 ampholines (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 9600 Vh (volt-hours). After equilibration in a buffer containing 10% glycerol, 50 mmol  $l^{-1}$  dithiothreitol, 2.3% SDS and 0.0625 mol  $l^{-1}$  Tris, pH 6.8, the tube gel was sealed to the top of a stacking gel that overlaid a 10% acrylamide slab gel (0.75 mm thick). SDS slab gel electrophoresis was carried out for 4 h at 12.5 mA/gel. Molecular mass standards varying from 14 to 220 kDa were added to the gel. Gels were stained with Coomassie Brilliant Blue R-250 to allow protein identification and quantification. For more details on methodology, refer to <http://www.kendricklabs.com/about2d.htm>.

### Protein spot identification and quantification

Protein spot profiles were analyzed from scanned 2-D gel images using PD\_Quest analysis software (Bio-Rad, Hercules, CA, USA). Matching of spots across gels and measurement of spot size in relation to total size of all spots within gels indicated that quantities for protein Spot 6 (Fig. 1), in particular, varied strongly between muscle samples from healthy and infected groups. The identity of Spot 6 was therefore examined using MALDI-TOF mass spectrometry followed by peptide mass fingerprinting. Coomassie-stained protein spots were excised from the gels, transferred to microcentrifuge tubes and destained by two alternating washes of 50 mmol  $l^{-1}$  aqueous ammonium bicarbonate and 100% acetonitrile. In-gel digestion was performed by addition of 0.1  $\mu$ g of trypsin (Promega, Madison, WI, USA), with digestion proceeding at 25°C for 24 h. Digestion products were extracted by washing three times with 1% formic acid in 50/50 (v/v) acetonitrile/water, and all extracts

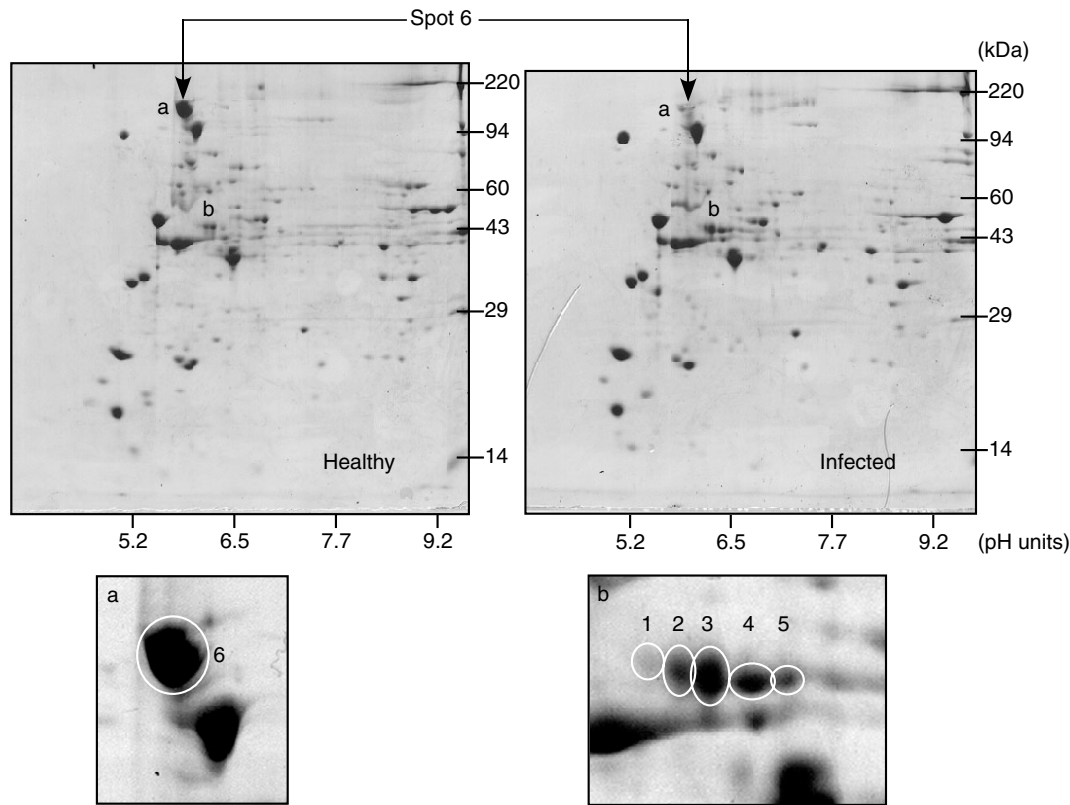


Fig. 1. Representative 2-D PAGE gels showing muscle protein expression in healthy and infected individuals. Lower panels highlighting spots of interest in areas 'a' (Spot 6) and 'b' (TnT Spots 1–5). Vertical dimension markers represent molecular mass (in kDa) markers added to the gel; horizontal dimension markers indicate pH range.

were combined and concentrated nearly to dryness using a Savant SpeedVac (Savant Instruments Inc., Farmingdale, NY, USA). The residue was diluted by addition of 50  $\mu\text{l}$  of 0.1% aqueous trifluoroacetic acid (TFA), and peptides were desalted using a C18 ZipTip (Millipore, Billerica, MA, USA) and eluted according to the manufacturer instructions. Digestion products were spotted onto the MALDI plate using alpha-cyano-4-hydroxycinnamic acid as matrix. Peptide mass mapping was performed on a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) in delayed-extraction reflectron mode using trypsin autolysis peaks as internal mass references (D. Jones, personal communication).

Tryptic digestion and MALDI-TOF analysis produced peptides whose masses were compared against entries in database NCBI nr.12.16.2006 constrained to *D. melanogaster* using MS-Fit routines within ProteinProspector (<http://prospector.ucsf.edu/>) (Clauser et al., 1999). One missed cleavage was allowed, and peptide mass tolerance was set to 50 p.p.m. Cysteines were assumed unmodified, and default amino acid modification mode was used.

The location of 4–5 TnT protein isoforms on 2-D gels was identified based on previous work that used a combination of gel electrophoresis and western blotting with an antibody against TnT (Marden et al., 1999). In the present study, in addition to spot 6, we examined normalized quantities for five TnT protein isoforms (Spots 1–5 in Fig. 1).

#### *Proteolytic digestion of purified dragonfly MHC*

To further examine the identity of Spot 6 (a proposed MHC degradation product; see Results), *L. pulchella* MHC was

purified and digested with mammalian calpain (rabbit skeletal muscle calpain 80 kDa subunit; P4533 Sigma-Aldrich, St Louis, MO, USA). Preparation of purified MHC was similar to that described previously (Johnson and Bennett, 1995). Briefly, basalar muscles were dissected out, homogenized in 0.5 mol  $\text{l}^{-1}$  KCl, 0.03 mol  $\text{l}^{-1}$   $\text{NaHCO}_3$ , pH 7.0 and incubated for 20 min at 4°C. Homogenates were centrifuged at 10 000  $g$  for 10 min at 4°C, after which the pellet was discarded. Supernatants were rapidly diluted in 1:15 v/v ice-cold  $\text{ddH}_2\text{O}$ . Suspensions were centrifuged at 10 000  $g$  for 10 min, after which supernatants were discarded. Pellets were suspended in 1 mol  $\text{l}^{-1}$  KCl and incubated for another 20 min at 4°C, centrifuged at 10 000  $g$  for 10 min and rapidly diluted in 1:15 v/v  $\text{ddH}_2\text{O}$ . Suspensions were centrifuged one last time at 10 000  $g$ , for 10 min, after which the supernatants were discarded and the pellet, now enriched in myofibrillar proteins, was dissolved in  $1\times$  gel buffer.

MHC purification from the enriched myofibrillar fraction was accomplished by separation using SDS-PAGE (4% stacking, 7.5% separating gel). Gels were briefly (30 s) stained with Coomassie Blue to visualize proteins and then washed with  $\text{ddH}_2\text{O}$ . MHC bands (~200 kDa) were cut out using a razor blade. Excised bands were finely chopped and homogenized in equal-volume 50 mmol  $\text{l}^{-1}$  Tris-HCl, 0.5% SDS, incubated at room temperature for 1 h, centrifuged for 15 min (10 000  $g$ ), after which supernatants were carefully removed.

Purified MHC samples were digested using a 0.3 mg  $\text{ml}^{-1}$  calpain [and 90  $\mu\text{l}$  calpain activating solution; preparation according to (Lakey et al., 1993)] solution for 45 min at 25°C. Digestions were stopped by adding equal-volume sample buffer and heating at 95°C for 2 min. Resulting peptides were resolved on a 4–20% SDS-PAGE gel and silver stained (Bio-Rad).

### Flight muscle performance

Animals were used in experiments within 4–6 h of capture. Maximum muscle mass-specific power output of the first basalar muscle was determined using a modification of the work-loop technique (Josephson, 1985) described previously (Marden et al., 2001; Schilder and Marden, 2004). Importantly, maximum muscle power output represents performance during imposed conditions that approximate *in vivo* contraction regimes. Muscle power output results were reported in an earlier publication (Schilder and Marden, 2006) and are used here to examine how power relates to muscle protein composition.

### Results

#### Identification of a MHC degradation product

Masses of 19 of the 35 peptides from Spot 6 (Fig. 1) obtained by trypsin digestion and MALDI-TOF analyses matched predicted trypsin digest fragment masses of *Drosophila melanogaster* muscle MHC (accession no. P05661; predicted molecular mass 224.5 kDa). This highly significant match (MOWSE score= $1.6 \times 10^{10}$ ) identifies the protein as MHC, but there is a large discrepancy between the molecular mass of *D. melanogaster* muscle MHC and that of Spot 6 on 2-D gels, as we determined the molecular mass of the latter to be between 150 and 164 kDa (analysis not shown; but see also Fig. 1). In addition, intact MHC cannot readily be resolved by 2-D gel electrophoresis, as it does not focus well in the first (i.e. IEF pH gradient) dimension (Reggiani and Te Kronnie, 2004; Gelfi et al., 2006). We therefore further examined the identity of Spot 6.

A group of  $\text{Ca}^{2+}$ -dependent cysteine proteinases known as calpains are implicated in the controlled degradation of myofibrillar proteins (e.g. Kim et al., 2005). Pemrick and Grebenau reported that proteolytic activity by calcium-activated neutral protease (CANP; also known as calpain) in the head region of ~200 kDa MHC from rabbit muscle produced 180,

165 and 150 kDa sized MHC peptides (Pemrick and Grebenau, 1984). Similarly, digestion of 200 kDa *Octopus* MHC by endogenous calpain-like proteinase CaDP results in a 155 kDa proteolytic product (Hatzizisis et al., 2000). A recent study indicated that protein digestion site preference of vertebrate calpains is highest where the amino acids lysine (K) and serine (S) occur at the P1 and P1' position (i.e. the amino acid positions between which calpain digests a protein) across a wide range of proteins (Tompa et al., 2004). In the *Drosophila* muscle MHC head region, there are two such sites (Lys<sup>559</sup>–Ser<sup>560</sup> and Lys<sup>611</sup>–Ser<sup>612</sup>) at which theoretical digestion by calpain results in 161 kDa and 155 kDa (calculated using tools at <http://pir.georgetown.edu/pirwww>) proteolytic products, respectively (Fig. 2A). Interestingly, all 19 peptide matches obtained from MALDI-TOF analyses of Spot 6 align within these two theoretical digestion products of *Drosophila* MHC (i.e. the regions marked black in Fig. 2). Moreover, the location of the two calpain digestion sites in the MHC head region appears to be conserved in insects (Fig. 2B). This led us to hypothesize that Spot 6 on our 2-D gels represents a calpain MHC degradation product.

Dragonfly (muscle) calpain has not been characterized, but muscle calpains share significant identity across phyla [i.e. invertebrates and mammals (Kim et al., 2005)]. We therefore used a mammalian skeletal muscle calpain (see Materials and methods; this rabbit skeletal muscle calpain 80 kDa catalytic subunit aligns with up to 43% amino acid identity and 62% similarity over its entire 422 amino acid length with the deduced amino acid sequence of a number of insect and crustacean calpains; tBLASTn, E value= $1 \times 10^{-92}$ ) to digest purified dragonfly MHC and examine the size of peptides

Fig. 2. (A) 3-D structure model of *Drosophila melanogaster* MHC head region (i.e. incomplete MHC), showing theoretical calpain digestion sites (marked as red and blue spheres). Regions that would be excluded from Spot 6 due to digestion by calpain are shown in grey. (B) Protein sequence alignment for three insect species of MHC head region in which theoretical calpain digestion sites are located. One such site (Lys<sup>560</sup>–Ser<sup>561</sup>) is present for the mosquito *Anopheles gambiae* (*A. gam*), while both sites are present for the fruitfly *Drosophila melanogaster* (*D. mel*) and honey bee *Apis mellifera* (*Ap. mel*; Lys<sup>561</sup>–Ser<sup>562</sup> and Lys<sup>613</sup>–Ser<sup>614</sup>, respectively).

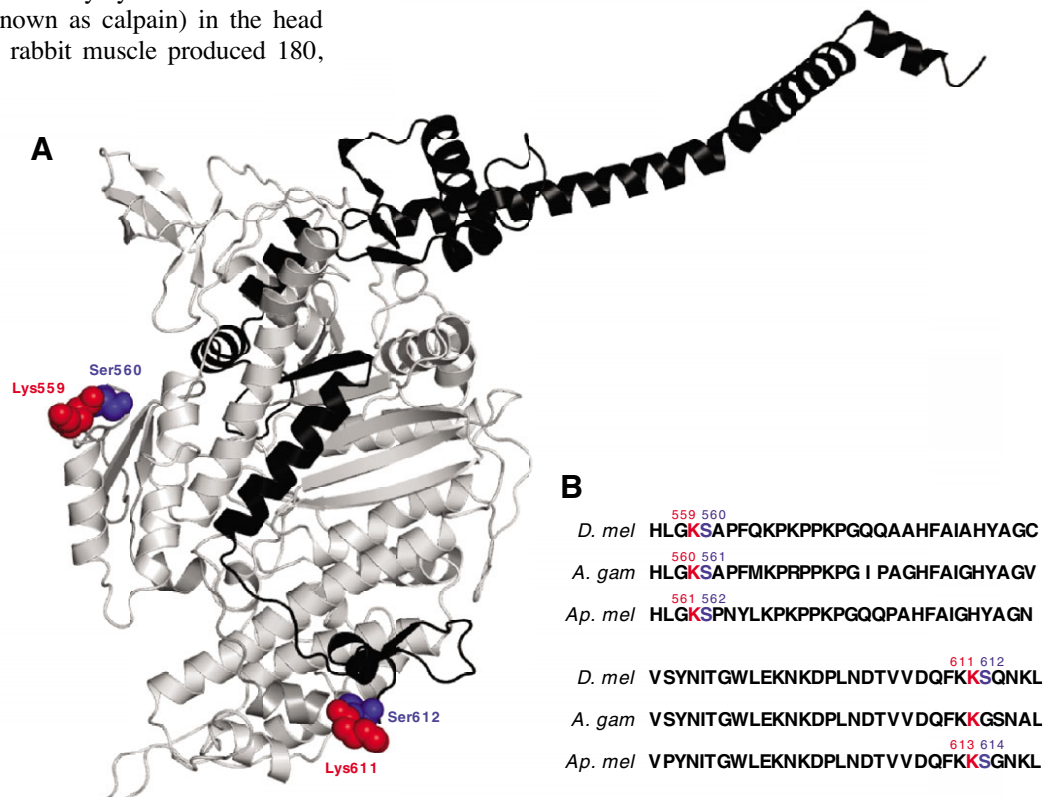


Fig. 3. (A) Western blot on muscle homogenates enriched in myofibrillar proteins from infected (i) and healthy (h) individuals, probed with a monoclonal antibody to *Drosophila* MHC. Lanes contain identical protein concentrations. Aside from the 206 kDa muscle MHC and non-specific reactivity to some high-molecular-mass proteins, a 165 and 155 kDa protein were identified. Left tick marks represent molecular mass standards (lane not shown). The presence of 165 and 155 kDa bands in infected muscle indicates that this may be a relatively 'high-powered' infected individual, as shown in Fig. 5E.

However, no performance measures were obtained from this individual's muscles. (B) Silver-stained SDS-PAGE gel showing peptides obtained by calpain (lane b) digestion of purified *L. pulchella* MHC from both healthy and infected individuals (lane a). In calpain-treated samples, a 156 kDa peptide can be observed, as well as a 122 and 110 kDa peptide. In addition, we putatively identified a calpain 80 kDa subunit, and other bands as calpain autodegradation products [i.e. similar to results reported by Pemrick and Grebenau (Pemrick and Grebenau, 1984)].

generated. The collection of peptides obtained after digestion of purified dragonfly MHC with rabbit calpain is visualized in Fig. 3B. Three of the bands are likely to be *in vitro* calpain degradation products of purified dragonfly MHC, with a prominent ~156 kDa band showing molecular mass similarity to our Spot 6 (Fig. 1). The numerous lower molecular mass bands observed in Fig. 3B appear to be the result of calpain autodegradation, as observed previously (Pemrick and Grebenau, 1984).

An alternative hypothesis for the cause of the difference in MHC155 abundance on 2-D gels between healthy and infected dragonflies is that there is an infection-associated change in MHC isoform expression and that native enzymatic digestion of different MHC isoforms results in a different collection of MHC-derived peptides (i.e. the hypothesis that Spot 6 disappears because its source isoform disappears). In our digestion experiment using rabbit calpain, healthy and infected individuals showed no differences for any of the bands present

on these gels (Fig. 3B), indicating that the loss of Spot 6 in infected dragonflies was not a result of a change in expression of MHC isoforms that differ in susceptibility to calpain proteolytic digestion.

We confirmed the presence of 206 kDa, 165 kDa and 155 kDa MHC proteins in dragonfly flight muscle homogenates by means of western blotting (see Fig. 3A), probing with a monoclonal *Drosophila* MHC antibody (generously provided by D. Kiehart). However, since we could not consistently resolve two separate entities within Spot 6 in our analyses of 2-D gels, we will from here onwards refer to Spot 6 as MHC155, a product of MHC degradation.

#### Myofibrillar protein expression comparison

We found significantly higher mean values ( $P < 0.001$ ) for the ( $\log_{10}$ -transformed) abundance (normalized quantities) of MHC155 for healthy than for infected individuals ( $0.60 \pm 0.22$  and  $-0.69 \pm 0.34$ , respectively) (Fig. 4A). This finding indicates that infection is associated with a significant decrease in dragonfly flight muscle MHC degradation.

Quantitative variation in TnT isoform composition was not affected by infection to the degree that MHC155 was, as we found no significant infection-related differences ( $P = 0.36$ ) between the means of the first principal component (PC1) of variation in relative abundance of TnT protein isoforms ( $1.05 \pm 1.59$  and  $0.51 \pm 0.81$  for healthy and

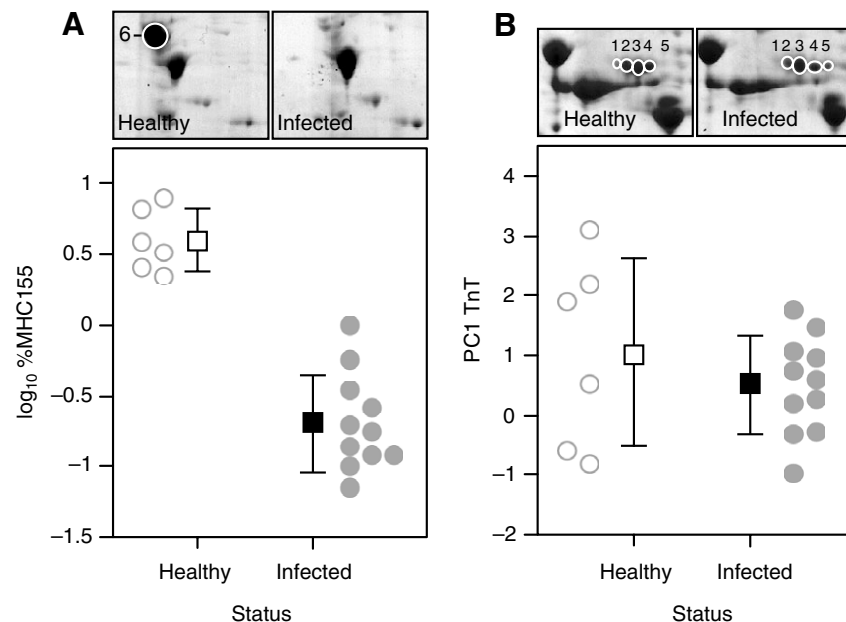
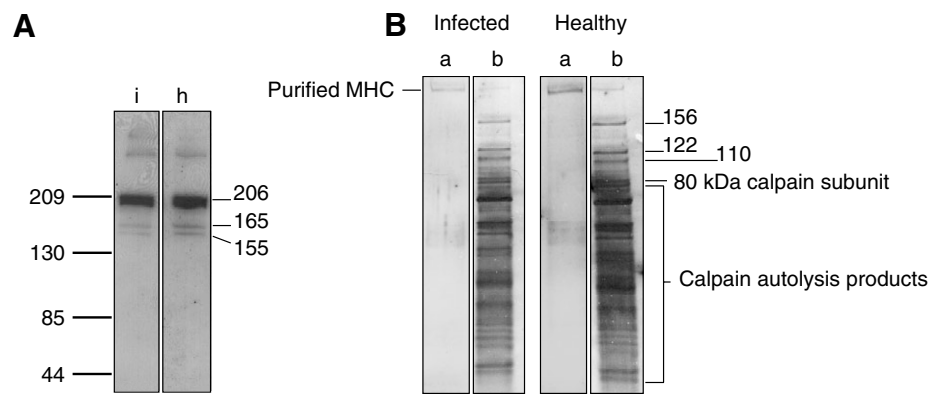


Fig. 4. Quantitative variation in MHC155 abundance and the first principal component (PC1) of TnT isoform composition in flight muscles of healthy ( $N = 6$ ) and infected ( $N = 11$ ) dragonflies. Upper panels highlight comparisons for (A) MHC155 and (B) TnT spots between healthy and infected individuals. Lower panels show comparison of mean values of  $\log_{10}$  %MHC155 and PC1 TnT. Error bars represent standard deviation from means.

infected, respectively) (Fig. 4B). Rather, TnT isoform composition appears to vary widely (as demonstrated by relatively large standard deviations) within both healthy and infected groups.

#### Flight muscle performance

We found previously that there is a significant difference in maximum flight muscle *in vitro* mechanical power output between healthy and infected dragonflies ( $148.6 \pm 23.1$  and  $117.1 \pm 32.0$  W kg<sup>-1</sup>, respectively;  $P=0.0002$ ,  $N=52$ ) (Schilder and Marden, 2006). In the current study, we used a subset of those muscles that spanned the range in observed power output. Among these 17 individuals, muscle power output varied from 88 to 184 W kg<sup>-1</sup> ( $N=6$ ) in healthy dragonflies and from 93 to 164 W kg<sup>-1</sup> ( $N=11$ ) in infected dragonflies. A Student's *t*-test revealed no significant difference for the contraction frequency at which maximum muscle power output was achieved for healthy *versus* infected dragonflies ( $40.3 \pm 1.5$  Hz and  $40.8 \pm 1.1$  Hz, respectively;  $P=0.80$ ).

#### Relating protein expression to muscle performance

Flight muscle performance of healthy individuals was to a large extent predicted by both MHC155 (Fig. 5A) and PC1 TnT (Fig. 5B). Within the healthy group, both MHC155 ( $r^2=0.85$ ,  $P=0.0089$ ) and PC1 TnT ( $r^2=0.79$ ,  $P=0.018$ ) were negatively correlated with muscle performance. This was not true for infected individuals. No significant correlation was found between muscle performance and either MHC155 (Fig. 5C) ( $r^2=0.26$ ,  $P=0.106$ ) or PC1 TnT (Fig. 5D) ( $r^2=0.01$ ,  $P=0.73$ ) for infected individuals.

When data for healthy and infected individuals were combined, we found a significant convex relationship ( $r^2=0.38$ ,  $P=0.03$ ) between muscle performance and MHC155 abundance (Fig. 5E). Low-powered healthy dragonflies showed increased muscle MHC155 whereas low-powered infected individuals had decreased MHC155 abundance. This result suggests that, with respect to flight muscle performance, there is an optimum MHC degradation rate. However, because our methods require destructive sampling, we lack time series data (i.e. muscle power *vs* MHC155 abundance over time) necessary to evaluate that hypothesis.

For healthy and infected individuals combined (Fig. 5F), PC1 TnT explained 31% of the variation in muscle performance ( $P=0.021$ ). Although data for TnT expression failed to explain variation in muscle performance among infected individuals separately (Fig. 5D), they fit along the same trend found for healthy individuals. Thus, flight muscle performance declines with increasing PC1 TnT and this relationship was not significantly affected by infection, other than to perhaps increase variation around the central tendency.

For healthy individuals, a multiple linear regression model including both MHC155 abundance and PC1 TnT explained

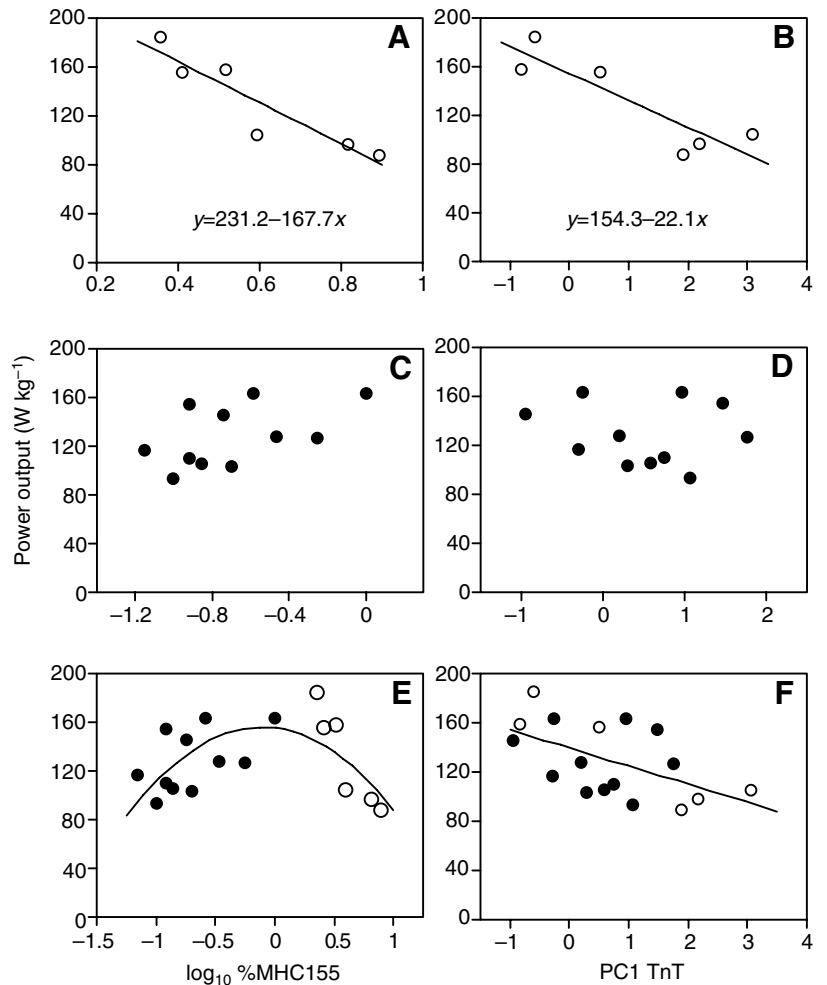


Fig. 5. Relationships between muscle power output (W kg<sup>-1</sup>) and (A) log<sub>10</sub> %MHC155 ( $r^2=0.85$ ) and (B) PC1 TnT ( $r^2=0.79$ ) obtained from principal component analysis of five TnT spot quantities for healthy dragonflies (open circles,  $N=6$ ). (C,D) No significant relationships between muscle power output and MHC155 and PC1 TnT values were found for infected individuals (filled circles,  $N=11$ ). Relationships between muscle power output and (E) log<sub>10</sub> %MHC155 [muscle power output =  $159.05506 + 16.421664(\log_{10}\%MHC155) - 55.997116(\log_{10}\%MHC155 - 0.2526)^2$ ] and (F) PC1 TnT [muscle power output =  $139.5 - 14.6(PC1TnT)$ ] for healthy (open circles) and infected (filled circles) individuals combined.

97.9% of variation in flight muscle power output (Fig. 6A) ( $P=0.0014$ ). Both predictors significantly contributed to the explanatory power of this model (see Table 1; log<sub>10</sub> %MHC155,  $P=0.0061$ ; PC1 TnT,  $P=0.010$ ). Although still significant (adjusted  $r^2=0.29$ ,  $P=0.034$ ), this model lost much of its explanatory power (98.7% to 38.3%) when data for infected individuals were included (Fig. 6B), and log<sub>10</sub> %MHC155 was no longer a significant predictor (see Table 1;  $P=0.21$ ). Because infection did not significantly affect TnT protein isoform composition (Fig. 4B, Fig. 5F), we conclude that infection reduces flight muscle contractile function primarily by affecting MHC degradation.

#### Discussion

To relate the observed changes in muscle protein composition to the reduction in mechanical power output, it is helpful to first

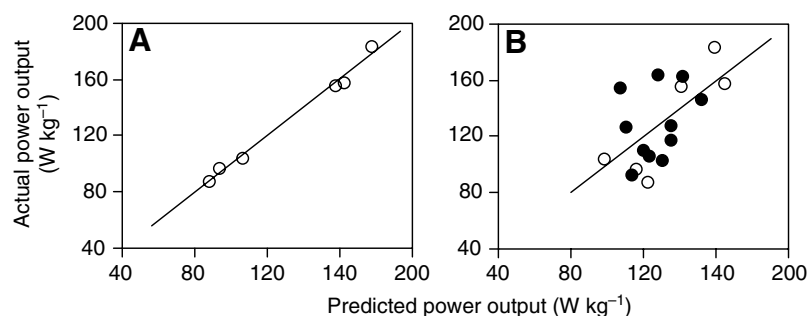


Fig. 6. Results of multiple linear regression of muscle power output ( $\text{W kg}^{-1}$ ) and the two predictor variables  $\log_{10}$  %MHC155 and PC1 TnT (see also Table 1), showing the relationship between actual muscle power output and that predicted by the two parameter model (adjusted  $r^2=0.979$ ,  $P=0.001$ ,  $N=6$ ) for healthy individuals (A). The same model explains only 38.3% (adjusted  $r^2=0.295$ ,  $P=0.034$ ,  $N=17$ ) of the variation in muscle power output when data for infected individuals (filled circles) are included (B).

consider that the quality of muscle contractile performance depends on a proper balance between mechanisms that maintain cytoskeletal integrity and control protein isoform expression and incorporation in sarcomeres. Studies that have examined variability of muscle contractile function at the cellular and molecular level have focused on only one side of these processes, thereby largely ignoring other important aspects of muscle cellular biology. Results presented here are a novel examination of how both myofibrillar protein isoform composition and protein degradation interact to affect variability in muscle performance.

Our results indicate that an important relationship exists between dragonfly flight muscle MHC degradation and TnT protein isoform composition. For healthy dragonflies, these two parameters combined to explain almost all (i.e.  $\sim 98\%$ ) variation in muscle contractile performance (albeit in a small sample). Thus, combining metrics of different aspects of muscle cytoskeletal composition and quality may provide higher explanatory power than limiting studies to only protein isoform expression, or to only protein turnover and cytoskeletal integrity.

It is important to note that we cannot be certain that the observed changes in MHC155 abundance and TnT protein isoform expression are the causative mechanisms for the observed variation in muscle power output, as these proteins may be markers for changes in a suite of co-regulated genes and/or proteins, including large sarcomeric proteins such as projectin that are not resolved on standard protein gels. However, we observed no apparent suite of correlated spot changes on our 2-D gels. Both MHC155 abundance and TnT isoform composition varied over a wide range, independently of each other and other proteins (Figs 1, 4).

#### MHC degradation

Based on 2-D gel spot identification by means of MALDI-TOF mass spectrometry, western blotting using MHC antibody, and calpain digestion experiments, we determined that MHC155 is most likely a degradation product of MHC and therefore a possible indicator of MHC turnover in dragonfly flight muscle. However, since we have not identified or examined any process *in vivo* relating to changes in MHC turnover, this conclusion and the following discussion are necessarily somewhat speculative.

Low-powered healthy individuals exhibited greater amounts of MHC155 (see Fig. 5A) and therefore perhaps a higher myosin degradation rate than high-powered healthy individuals. One hypothesis is that the amount of MHC degradation may be related to the nutritional status of dragonflies. Low nutritional status may induce increased MHC proteolysis to mobilize amino acids for use as energy substrates. This could create a net loss of MHC in the flight muscles and a reduction in contractile performance. Indeed, low-energy (fasting) conditions have been associated with increases in muscle proteolysis in rats (Kettelhut et al., 1994) and increased expression levels of calpains responsible for myofibrillar degradation in rainbow trout (Salem et al., 2005).

Alternatively, variation in abundance of MHC155 may be a result of age differences within the healthy group of dragonflies. Aging vertebrate muscle is commonly characterized by the loss of muscle function (Stuerenburg et al., 2006; Karakelides and Sreekumaran Nair, 2005) and imbalances between myofibrillar protein synthesis and degradation (e.g. Yarasheski, 2003). The observed changes in MHC155 abundance were an unanticipated result and therefore we did not obtain measures of nutritional status or age for the individuals used in this study. We highlight

Table 1. Parameter estimates and standard errors for multiple linear regression models (see also Fig. 6) of response variable muscle power output ( $\text{W kg}^{-1}$ ) and predictors  $\log_{10}$  %MHC155 and PC1 TnT for healthy individuals only and healthy + infected individuals combined

	Term	Estimate	s.e.	t-ratio	P-value
Healthy	Intercept	208.7	8.3	25.12	<b>0.0001</b>
	$\log_{10}$ %MHC155	-108.1	15.5	-6.96	<b>0.006</b>
	PC1 TnT	-12.3	2.1	-5.80	<b>0.01</b>
Healthy + infected	Intercept	144.4	8.1	17.8	<b>&lt;0.0001</b>
	$\log_{10}$ %MHC155	12.5	9.5	1.31	0.21
	PC1 TnT	-17.4	5.9	-2.93	<b>0.01</b>

Values in bold indicate significance at  $\alpha=0.05$ .



these possibilities for the purpose of shaping future hypotheses rather than as well-supported explanations of the present results.

#### TnT isoform expression

Variability in TnT protein isoform composition is at least partly determined by alternative splicing of the *troponin-t* gene. This mechanism drives variability in the relative abundance of distinct *troponin-t* mRNA transcripts, which has previously been shown to correlate with muscle contractile and flight performance (Fitzhugh and Marden, 1997; Marden et al., 1999; Marden et al., 2001). The relationship between *troponin-t* splicing and muscle performance is strengthened by the present results, which demonstrate for the first time a quantitative relationship between muscle power output and TnT isoform composition at the protein level (Fig. 5B,F).

The first principal component of TnT protein isoform composition had a strong positive correlation with the two most abundant TnT spots (i.e. Spots 2 and 3), as indicated by their component loading scores (Table 2). The most abundant TnT spot (i.e. Spot 2) showed a negative correlation with muscle power output (results not shown), which is similar to findings for RNA level analyses in which the *troponin-t* transcript that had the highest relative abundance also had a negative relationship with muscle power (Marden et al., 2001). As explained in that paper, relative abundances of isoforms are interrelated, with increases in relative abundance of particular isoforms necessarily accompanied by decreases in relative abundance of others. For that reason, it remains difficult to determine if variation in contraction is affected by increases in isoforms that promote contractility or by decreases in isoforms that inhibit contractility.

#### Effects of gregarine infection

Natural infection by gregarines of the dragonfly midgut impairs flight muscle contractile performance and disrupts the apparent relationship between MHC155 abundance and muscle power output. This infection-associated change in muscle protein composition appears to be a specific effect on myosin rather than general effects across all muscle proteins, as evidenced by the overall similarity of 2-D gel spot patterns and the fact that PC1 TnT does not markedly differ between healthy and infected individuals, whereas MHC155 abundance decreases radically (Fig. 4A).

We previously reported that gregarine infection causes chronic inflammation of *L. pulchella* flight muscles and

metabolic disturbances that include a switch in substrate utilization towards glycolytic metabolism, loss of lipid oxidation ability and deposition of lipid around flight muscles, and an impaired response to exogenous insulin (Schilder and Marden, 2006). These findings are particularly interesting with respect to the decrease in MHC degradation found for infected individuals. Mammalian obese and insulin-resistant phenotypes that are commonly associated with chronic inflammation (Wisse, 2004) have recently been linked to a decreased expression of *calpain 3* (Walder et al., 2002). *Calpain 3* expression is specific to skeletal muscle and is thought to be involved in myofibrillar protein degradation during muscle remodeling (Kramerova et al., 2005) and metabolic signaling pathways (Walder et al., 2002). In light of these mammalian symptoms, and based on the assumption that MHC155 is a calpain degradation product, we tentatively hypothesize that decreased expression of a calpain ortholog in dragonflies underlies the observed decrease in MHC degradation that is associated with infection-induced metabolic disease.

Alternatively, the decrease in MHC155 in muscle of infected dragonflies might be a result of parasite-induced changes in behavior and muscle usage. Flight muscles of *L. pulchella* males infected with gregarines are unable to oxidize lipids and they tend to switch from energetically costly territorial behavior to a cheaper (much less flying) 'satellite lifestyle' (Marden and Cobb, 2004). This behavioral change may be directly linked to the muscle's inability to use lipid fuels (Schilder and Marden, 2006), and reduced flight duration and intensity may decrease the rate of myosin turnover. In this light, the differences in MHC degradation between healthy and infected individuals could be an effect of training or recent experience.

#### Perspective

Skeletal muscle is an intricate and precise machine that is capable of modulating its performance to very subtle changes in physiology (Clark et al., 2002). An emerging picture from our research is that, in addition to changes in TnT splicing that serve to adjust muscle contractile performance (Marden et al., 2001), dragonfly flight muscles also show functionally important variation in MHC processing that varies according to parasite infection and combines with TnT isoform profile to explain most of the variation in muscle performance in healthy dragonflies. Presence of non-invasive protozoans in the midgut lumen causes dramatic changes in muscle energy metabolism, protein composition, a molecular marker of inflammation, and

Table 2. Mean abundance (percentage of total optical density of all protein spots on a gel) and component loading scores for individual TnT protein spots used in the principal component analysis that generated three independent principal axes (PC1–PC3 TnT)

	Mean abundance (%)	PC1 TnT component loading score	PC2 TnT component loading score	PC3 TnT component loading score
TnT Spot 1	0.97±1.45	0.090	0.344	-0.758
TnT Spot 2	2.25±2.62	<b>0.686</b>	0.277	0.056
TnT Spot 3	2.14±1.62	<b>0.713</b>	-0.202	0.021
TnT Spot 4	0.91±0.55	0.111	-0.727	0.086
TnT Spot 5	0.52±0.70	0.008	0.486	0.643

Values in bold show the strong positive correlation of TnT Spots 2 and 3 with PC1 TnT (see Discussion).

behavior. Metabolic pathologies of infected dragonflies are highly similar to those associated with mammalian metabolic syndrome and obesity (Schilder and Marden, 2006), and in the present study we find another parallel – a decrease in myosin degradation that may be caused by a reduction in calpain expression, as has been reported in obese and insulin-resistant mammals (Walder, 2002).

The effect of parasites on insect muscle is a nascent avenue of research with many uncertainties yet to be resolved, but an emerging picture is that the pathologies observed are highly congruent to those that occur in mammalian metabolic diseases. Invertebrates provide accessible systems for studying basic biology of muscle and yield results and hypotheses that may compliment, inform and stimulate research on muscle biology and metabolic diseases in general.

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