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Flight duration and flight muscle ultrastructure of unfed hawk moths

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ABSTRACT

Flight muscle breakdown has been reported for many orders of insects, but the basis of this breakdown in insects with lifelong dependence on flight is less clear. Lepidopterans show such muscle changes across their lifespans, yet how this change affects the ability of these insects to complete their life cycles is not well documented. We investigated the changes in muscle function and ultrastructure of unfed aging adult hawk moths (*Manduca sexta*). Flight duration was examined in young, middle-aged, and advanced-aged unfed moths. After measurement of flight duration, the main flight muscle (dorso-longitudinal muscle) was collected and histologically prepared for transmission electron microscopy to compare several measurements of muscle ultrastructure among moths of different ages. Muscle function assays revealed significant positive correlations between muscle ultrastructure and flight distance that were greatest in middle-aged moths and least in young moths. In addition, changes in flight muscle ultrastructure were detected across treatment groups. The number of mitochondria in muscle cells peaked in middle-aged moths. Many wild *M. sexta* do not feed as adults; thus, understanding the changes in flight capacity and muscle ultrastructure in unfed moths provides a more complete understanding of the ecophysiology and resource allocation strategies of this species.

1. Introduction

Flying insects rely on flight for a multitude of essential functions, including finding food or mates, dispersing, or avoiding predators. However, flight is not a lifelong requirement in several orders of insects such as the Diptera (Hocking, 1952), Coleoptera (Jackson, 1933; Chapman, 1956), Heteroptera (Brinkhurst, 1959; Edwards, 1969; Andersen, 1973; Solbreck, 1986; Kaitala, 1988), Homoptera (Johnson, 1953, 1976; Kobayashi and Ishikawa, 1993), Hymenoptera (Janet, 1906, 1907; Jones et al., 1978), and Orthoptera (Readym and Josephson, 1982; Shiga et al., 1991; Tanaka, 1991, 1993; Tanaka and Suzuki, 1998). For such insects, flight muscle breakdown is associated with reproduction. In many of these species, adults emerge with fully developed flight muscles, which subsequently break down before or during the reproductive stage (Johnson, 1976; Zera and Denno, 1997; Marden, 2000) and flight is only required for the pre-reproductive migratory or dispersal phase in early adult life before initiating reproduction (Johnson, 1969; Harrison, 1980). However, in insect orders such as the Lepidoptera (Ziegler, 1991; Stjernholm et al., 2005; Jervis et al., 2005; Boggs, 2009) with lifelong dependence on flight, it is not clear if or when flight muscle breakdown occurs.

Studies of adult lepidopterans suggest that muscle breakdown might occur in many of these species (Karlsson, 1994, 1998; Stjernholm and Karlsson, 2000; Norberg and Leimar, 2002; Stjernholm et al., 2005; Stjernholm and Karlsson, 2008; Åhman and Karlsson, 2009), but whether there is degeneration of muscle function and capacity over the entire lifespan of these insects is not clear (Stjernholm and Karlsson, 2008). Recently, Niitepõld et al. (2014) showed that body mass, flight, and peak metabolic rates decrease with age in the lepidopterans *Speyeria mormonia* and *Colias eurytheme*, suggesting that muscle mass and function decrease with age. Still, the exact ways in which aging might affect flight capacity in flying insects are not known. Stjernholm et al. (2005) reported intriguing age-related reallocation of resources from flight muscle to reproductive organs with no adverse effects on flight capacity under natural conditions in lepidopterans. In contrast, amino acids from nectar are allocated to flight muscles when moths have the opportunity to feed (Levin et al. 2017 a, b), suggesting that maintenance of flight capability is prioritized when possible. Interestingly, the body masses of hawk moths continuously decrease

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whether or not they are feeding (Ziegler, 1991). If lepidopterans do experience decreased muscle mass across their lifespan, how might such muscle breakdown affect their ability to fly and complete their life cycle?

We investigated the changes in muscle function and muscle ultrastructure during the lifespan of unfed hawk moths. This moth species lives in variable environments with the potential for frequent episodes of starvation (Davidowitz, 2002; Alarcón et al., 2008; Contreras et al., 2013; Levin et al., 2016) and flight is an essential component of their life history. Both male and female moths hover when probing flowers for nectar, which can be a metabolically demanding mode of locomotion (Bartholomew and Casey, 1978; Lehmann and Dickinson, 1997). In addition to requiring flight to feed, males need to fly to find females (Levin et al., 2016), and females need to fly and hover to oviposit. Our study asked whether age affects flight capacity in starved *Manduca sexta*, what changes might occur in flight muscle ultrastructure as *M. sexta* ages, and how such changes might affect flight capacity.

2. Materials and methods

2.1. Experimental animals

Carolina sphinx moths (Lepidoptera: Sphingidae: *M. sexta* (hawk moths)) from the colony at the University of Arizona, Tucson, AZ were used for all experiments. Larvae were reared on an *ad lib* artificial diet (Davidowitz et al., 2003) and once eclosed, adults were reared for the experiments described below. For the flight capacity and muscle ultrastructure experiment, adults were reared individually in a small 28 cm L × 28 cm W × 28 cm H, 299 cm^3 cage (BioQuip Products, Rancho Dominguez, CA, USA). These cages were kept in a reversed light/dark cycle of 16:8 h maintained at 27 ± 2 °C and 40–45% RH. Moths were not fed to avoid any possible influence of adult-derived nutrients (Arrese and Soulages, 2010; Gondim et al., 2013; Levin et al., 2017a; b) on any changes in flight capacity and muscle ultrastructure. Moreover, recent studies have shown that wild-caught adult *M. sexta* routinely experience limited nectar resources: 15% did not feed at all and 73% did not feed on their preferred nectar resources (Levin et al., 2016). Similarly, laboratory-reared *M. sexta* routinely do not feed (Ziegler, 1991), as indicated by their metabolic profiles across diel time and age (Wone et al., 2018). Because the average lifespan of unfed female *M. sexta* is 8.0 d and the average lifespan of unfed male *M. sexta* is 4.9 d (Wone et al., 2018), we divided the moths into three age groups: Day 1 (young), Day 3 (middle-aged), or Day 6 (advanced-aged) moths to determine the changes in flight muscle across their lifespan, with a total of $n = 5$ moths per sex per age group, for a total of n = 30 moths.

2.2. Flight capacity measurements

We used a custom-built computer-monitored flight mill to evaluate the flight capacity of *M. sexta,* because flight mills provide exact measurements of flight distance, speed, and duration (Thomas and William, 1992; Cui et al., 2013; Hao et al., 2013; Chen et al., 2015). Flight mills do not provide good estimates of free flight performance (Riley et al. 1997), however, they are considered a standard approach for comparing the effects of treatments when investigating flight behavior in insects (Schumacher et al., 1997; Ishiguri and Shirai, 2004; Tu et al., 2010; Chen et al., 2015). To prepare moths for the flight capacity measurements, newly eclosed moths were gently handled and scales were removed from an area of less than 4 mm^2 on the dorsal thoracic region of each moth's body. A drop of Loctite® Super Glue Gel (Henkel Corporation, Westlake, OH, USA) was used to glue a small metal plate (approximately 3 × 2 mm) onto the bare pronotum of each moth. The small plate was then attached to the arm of a flight mill with a magnet. Flight capacity, measured as distance flown, was measured only once on each moth on either Day 1, Day 3, or Day 6 depending on their treatment group ($n = 30$, 10 samples per age group). These nocturnal moths were flown during the dark cycle when their flight activity was greatest. The moths were allowed to fly on the mill for as long as they wanted, but if they stopped flying for more than 0.5 h the flight trial was ended and distance flown was recorded. The computer-monitored flight mill setup was maintained at a temperature of 27 ± 1 °C and RH between 35% and 40%.

2.3. Histological preparation

After measuring flight capacity, the main flight muscles (dorso-longitudinal muscle) were removed and histologically prepared for transmission electron microscopy (TEM) following Ribi (1987). In brief, flight muscles were longitudinally sectioned from moths in each age treatment ($n = 30$, five females and five males per age group) and fixed in 0.2 M piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), 25% glutaraldehyde, 16% paraformaldehyde, saturated picric acid, and distilled water for 1 h. Double-distilled water was used in all washes and rinses. Each fixed muscle was then washed in 0.1 M PIPES twice for 20 min. Post-fixation of each sample was performed in 0.2 M PIPES with 4% osmium tetroxide and distilled water to preserve lipids in each sample. After 30 min, each sample was rinsed and left overnight in 0.1 M PIPES. A solution of 10% acetone and 2% aqueous uranyl acetate in distilled water was used to stain the muscle tissues. Dehydration of the prepped tissues was carried out through a series of 25%, 50%, 70%, and 90% aqueous ethanol solutions for 10 min each. Each specimen was then dehydrated again three times in 100% ethanol for 40 min per incubation. Each sample was infiltrated first in 75% propylene oxide with 25% embedding resin overnight, then in 25% propylene oxide with 75% embedding resin for 6 h, and finally in 100% embedding resin overnight. The samples were embedded in longitudinal orientation in 100% ACLAR® sheets (Ted Pella, Inc., Redding, CA, USA) and polymerized at 80 °C for 36 h with care taken that all samples were placed in the same orientation. Thin sections (~200 μm) of embedded samples were then cut longitudinally using an ultramicrotome and were later stained with lead to enhance contrast between different cellular structures. Three slides were prepared from mid-longitudinal sections of each flight muscle for each moth.

We evaluated flight muscle ultrastructures including the number of mitochondria, size of mitochondria, percent area of mitochondria, number of sarcomeres, percent area of sarcomeres, and length of sarcomeres using TEM imaging. Micrographs were recorded on a CM-12 TEM at 8800 × magnification (Philips, Andover, MA, USA). TEM images were initially processed using Photoshop CS5 software (Adobe Systems Incorporated, San Jose, CA) and then muscle ultrastructures were quantified using Image J v1.45 (Abràmoff et al., 2004). The value of each measurement for each sample was the average of 30 randomly chosen images at 8800 × magnification from three slides per moth (10 randomly chosen images at 8800 × magnification per muscle slide). All reported measurements from the TEM image of each sample are within the field of view in an area of 174 μm^2 at 8800 × magnification.

2.4. Statistical analysis

MANOVA was performed in RStudio (RStudio Team, 2015) to analyze any multivariate effects in the number of mitochondria, size of mitochondria, percent area of mitochondria, number of sarcomeres, percent area of sarcomeres, length of sarcomeres, and distance flown, with age and sex as factors. A significant MANOVA was followed by separate ANOVA performed in RStudio (RStudio

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