Flight duration and flight muscle ultrastructure of unfed hawk moths

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A B S T R A C T

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 life spans, 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 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 (dorsolongitudinal 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 muscles 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.

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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; Solbrec, 1986; Kaitala, 1988), Homoptera (Johnson, 1953, 1976; Kobayashi and Ishikawa, 1993), Hymenoptera (Janet, 1906, 1907; Jones et al., 1978), and Orthoptera (Ready 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 from 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 (Karls0n, 1994, 1998; Stjernholm and Karlsson, 2000; Norberg and Leimar, 2002; Stjernholm et al., 2005; Stjernholm and Karlsson, 2008; Ähman 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, Niitepold et al. (2014) showed that body mass, flight, and peak metabolic rates decrease with age in the lepidopterans Speyeria mormonia and Collas 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.
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; Alarcon 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 cm³ 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 diet 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; Gui 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 mm² 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 (dorsolongitudinal 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% acetic acid and 2% aqueous uranyl acetate in distilled water was used to stain the muscle tissues. Dehydration of the prepared 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% ACLA® resin (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 (Abramoff 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 µm² 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
3. Results

3.1. Flight capacity

MANOVA indicated significant multivariate effects for number of mitochondria, size of mitochondria, percent area of mitochondria, percent area of sarcomeres, length of sarcomeres, and distance flown, and age and sex as factors. Adult body mass was not included as a covariate to possibly account for body size effects on muscle ultrastructure (i.e., number of mitochondria), because the microscopic area of muscle tissue examined was standardized across all individuals. At this microscopic level (i.e., cellular-level), body size effects on the number of mitochondria are not expected because the number of mitochondria is well regulated in any given cell type (Robin and Wong, 1988). Individual univariate models were evaluated post hoc by pairwise comparisons using Tukey’s HSD (Honestly Significant Difference). To determine whether flight capacity (i.e., flight distance) is related to number of mitochondria, size of mitochondria, percent area of mitochondria, number of sarcomeres, percent area of sarcomeres, or length of sarcomeres, these variables were regressed on distance flown. Effect size for the individual linear regressions was calculated using Cohen’s $f^2$ (Cohen, 1988).

3.2. Muscle ultrastructure

The number of mitochondria in flight muscle changed with age ($F_{2, 27} = 91.2, p < 0.0001$). *M. sexta* collected on Day 3 post-eclosion had significantly greater numbers of mitochondria compared to moths collected on Day 1 or Day 6 (Figs. 1B and 3). The size of mitochondria in the flight muscle changed with age as well ($F_{2, 27} = 224.2, p < 0.0001$). The average size of mitochondria was significantly larger in Day 6 post-eclosion moths than in Day 1 or Day 3 moths (Fig. 1C). In addition, percent area of mitochondria increased as moths aged ($F_{2, 27} = 44.3, p < 0.0001$). Specifically, percent area of mitochondria was much lower in Day 1 post-eclosion moths compared to Day 3 or Day 6 moths (Fig. 1D). No effect of sex on the number, size, or percent area of mitochondria in flight muscle was detected.

The number of sarcomeres in the flight muscle changed with age ($F_{2, 27} = 28.6, p < 0.0001$). *M. sexta* collected on Day 1 post-eclosion had significantly fewer sarcomeres compared to those collected on Day 3 or Day 6 (Fig. 1E). Percent area of sarcomeres increased as the moths aged ($F_{2, 27} = 27.3, p < 0.0001$). Notably, percent area of sarcomeres was much lower in Day 1 post-eclosion moths compared to Day 3 or Day 6 moths (Fig. 1F). The length of the sarcomeres in the flight muscle also changed with age ($F_{2, 27} = 80.2, p < 0.0001$). Sarcomere lengths were greatest in Day 3 post-eclosion moths compared to Day 1 or Day 6 moths, whereas sarcomere length was shortest in Day 1 moths compared to Day 3 or Day 6 moths (Fig. 1G). No effect of sex on the number, percent area, or length of sarcomeres in the flight muscle was detected.

4. Discussion

Here, we provide the first detailed analysis of changes in flight capacity and flight muscle ultrastructure in unfed adult *M. sexta* over their lifespan. The present study provides insights into how flight capacity and muscle ultrastructure change over time in *M. sexta*. Such changes might affect the ability of moths with a lifelong dependency on flight to find food, mates, and oviposition sites while experiencing potentially long or frequent episodes of starvation.

4.1. Flight duration

Muscle function and strength have been linked to age (Demontis et al., 2013a; b), as we have also shown in *M. sexta* (Wone et al., 2018). Flight duration in Day 1 post-emergence moths was minimal and was maximal in Day 3 post-emergence moths. There was a significant increase in flight duration in Day 3 and this is positively correlated with flight muscle ultrastructure. These results are consistent with those from other flying insects, such as the honeybee (Herd, 1965), housefly (Soahal et al., 1972; Sohal, 1976), green-veined white butterfly Pieris napi (Ahman and Karlsson, 2009), and Drosophila (Sohal, 1975; Riddell and Ritzmann, 2005; Kim et al., 2011; Demontis et al., 2013a, 2013b). In other species, such as mice and humans, muscle function and strength decline with age (Carmell et al., 2002; Haddad and Adams, 2006) and these changes are thought to be related to changes in signaling pathways (Haddad and Adams, 2006), increased abundance of cytoskeletal proteins (Piec et al., 2005; O’Connell et al., 2007), and mitochondrial dysfunction (Tribe and Ashhurst, 1972; Yarian and Sohal, 2005; Heppe, 2014). Our results show that flight distance was significantly shorter in advanced-aged moths (Day 6) compared to middle-aged moths (Day 3), which can be partially explained by the significantly lower number of mitochondria in the flight muscle cells of advanced-aged moths compared to middle-aged moths. This would suggest that the ability of unfed *M. sexta* to find mates and suitable habitat to complete their life cycle is greatest within 3–4 days post-eclosion. Indeed, hawk moths are large, powerful fliers that can reach speeds of 5–10 km/h (Willmott and Ellington, 1997) and can fly distances greater than 20 km (Dawidowitz, unpubl. data). Unfed moths flew up to 3200 m and averaged 2903.5 m (±271.23 STD) on day 3 post-eclosion, but could still fly 600 m on day 6 (average 569.6 m ± 151.78 STD).

The results of the present study have implications for our understanding of the ecophysiological aspects of the life history of hawk moths. The adults used in the current study were raised in colonies that have been maintained under laboratory conditions at the University of Arizona since 1985 (Duch et al., 2000) in cages of about 1 m x 1 m x 1 m, and thus do not need to fly farther than 1 m
to find a mate, to feed, or to lay eggs. Moreover, adult *M. sexta* in this colony, especially males, fed irregularly whether nectar was available freely or individually hand fed (Ziegler, 1991; Contreras et al., 2013; Wone et al., 2018). Ziegler (1991) reported that out of 40 males, 10 moths fed only once and only one moth fed twice during their lifetime. In contrast, 33 of 40 female moths fed from one to six times during their lifetime. This difference in feeding behavior suggests that males normally do not feed. Wone et al. (2018) recently reported that the absence of diel shifts in the metabolic profiles of male moths indicates that males generally do not feed, whereas females generally do feed. In addition, Ziegler (1991) observed that male moths are much more active than females, suggesting that in males, the drive to reproduce is much greater than the drive to feed. Furthermore, males mate up to six times and females only once (Levin et al., 2016). These observations, combined with results from the current study, indicate that as long as males are able to find females within 3–4 d post-eclosion, they could mate even without having fed. During this relatively brief active period, wild adult moths in the Southwest US feed on the nectar of flowering *Datura wrightii* and *Agave palmeri*, which may be of limited availability in their environment (Alarcon et al., 2008, 2010; Raguso et al., 2003; Riffell et al., 2008). Hence, although wild adult moths can experience periods of starvation (Levin et al., 2016), at least the males do not need to feed in order to find mates and complete their life cycle. However, more importantly, because moth eggs contain ~70% water, females must drink (feed) more to maintain viable eggs (Kawooya and Law, 1988).

### 4.2. Muscle ultrastructure

The changes in flight muscle ultrastructure, including the fusion of mitochondria, reported in the present study are consistent with those observed in other flying insects. The fusion of mitochondria during aging has also been reported in flight muscles of other flying insects such as the housefly (Sohal et al., 1972; Sohal, 1976), black carpet beetle (Butler and Nath, 1972), *Drosophila* (Sohal, 1975; Miller et al., 2008), and the blowfly (Gregory et al., 1968). We observed a significant decrease in the number of mitochondria in unfed advanced-aged moths (Day 6) compared to unfed middle-aged moths (Day 3). As expected, by Day 6 the flight muscle had deteriorated, as indicated by the enlarged mitochondria (Fig. 3C). This increase in mitochondrial size is likely due to fusion of mitochondria (which greatly reduces oxidative phosphorylation) and is one of the age-related changes also observed in other animals (Seo...
Fig. 2. Relationship between distance flown and a subset of flight muscle ultrastructure in unfed Manduca sexta of different age classes. (A) Number of mitochondria and distance of flight. (B) Length of sarcomere and distance of flight. (C) Percent area of mitochondria and distance of flight. (D) Percent area of sarcomere and distance of flight. (E) Number of sarcomere and distance of flight. Further statistics are given in the text. Note: All the individuals that flew more than 2500 m were Day 3 moths, whereas Day 1 and Day 6 moths all flew less than 800 m. Unfilled diamonds – Day 1, Triangles – Day 3, and Circles – Day 6. Number of mitochondria, percent area of mitochondria, and percent area of sarcomere are measured in a 174 µm² area.
Mitochondrial fusion affecting flight capacity might have accelerated under unfed conditions in the current study. Fusion of aging mitochondria is thought to affect mtDNA integrity and respiratory function (Chen et al., 2005) and is controlled by the optic atrophy 1 (Opal) gene and the two GTPases mitofusin 1 and 2 (isoforms Mfn1 and Mfn2) in humans (Chan, 2006; Benard and Karbowski, 2009; Peterson et al., 2012). Other changes in flight muscle ultrastructure detected in unfed M. sexta include sarcomere and myofibril alterations. We found significant decreases in sarcomere size and myofibril changes and disorganization in the flight muscle of unfed aged M. sexta (Fig. 3C). Such changes are thought to be associated with a decrease in both muscle fiber types (Tomonaga, 1977; Scei et al., 1980; Kaminska et al., 1998), as well as their reduced function (Pastoret and Debille, 1995; Kirkendall and Garrett, 1998; Deschenes, 2004). Decreases in muscle fiber types likely explain the age-related muscle mass reduction seen in animals (Deschenes, 2004; Demontis et al., 2013a; Nilwik et al., 2013). Interestingly, sarcromere numbers and area increase after Day 1 are likely attributed to additional biosynthesis post-eclosion (Sohal, 1994).

Changes in muscle ultrastructure show how the flight muscle breaks down with age, which results in decreased muscle size and flight duration over the lifespan of aging lepidopterans. Decreasing number, size, and length of sarcomeres in M. sexta flight muscle at Day 6 post-eclosion all contribute to decreased flight capacity and muscle mass. Indeed, the total weights of fed or unfed adult M. sexta continuously decrease during their adult lifespan (Ziegler, 1991). One likely reason for the decrease in sarcomere length is age-related decreases in muscle fiber types (Deschenes, 2004; Demontis et al., 2013a; Nilwik et al., 2013). Moreover, as mentioned above, body mass also decreases with age in S. mormonia and C. eurhythme (Niiitpoldi et al., 2014), as well as in P. napi (Stjernholm and Karlsson, 2008). However, some lepidopterans show no adverse effect of age on flight capacity (Stjernholm et al., 2005). Such discrepancies likely reflect differing lifespans or life histories among species. That is, the longer-lived species might not show the muscle breakdown and concomitant decrease in flight capacity that is observed in the shorter-lived species such as M. sexta. Further studies are needed to identify any distinctions in flight muscle ultrastructure and function during aging between long- and short-lived lepidopteran species.

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