THE EFFECTS OF AGE AND LIFETIME FLIGHT BEHAVIOR ON FLIGHT CAPACITY IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

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by Steven J. Lane

The effects of flight behavior on physiology and senescence may be profound in insects due to the extremely high metabolic costs of flight. Previous studies show that flight capacity in insects decreases with age, and that limiting flight behavior extends lifespan and slows the age-related loss of antioxidant capacity and accumulation of oxidative damage in flight muscles. In this study, we tested the effects of age and lifetime flight behavior on flight capacity by measuring wingbeat frequency, the ability to fly in a hypo-dense gas mixture, and metabolic rate in *Drosophila melanogaster*. Specifically, 5-day old adult flies were separated into three life-long treatments: (A) those not allowed to fly (no flight), (B) those allowed – but not forced – to fly (voluntary flight), and (C) those mechanically stimulated to fly (induced flight). Flight capacity senesced earliest in flies from the no-flight treatment, followed by the induced-flight group and then the voluntary flight group. Wingbeat frequency senesced with age in all treatment groups but was greatest in the voluntary and induced flight groups. Metabolic rate during agitated flight senesced earliest and most rapidly in the induced flight group, and was low and uniform throughout age in the no flight group. Early senescence in the induced flight group was likely due to the accelerated accumulation of damage at the cellular level, while the early loss of flight capacity and low metabolic rates in the no-flight group demonstrate that disuse effects also significantly alter senescence patterns of whole-insect performance.

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DEFINITION OF TERMS

FMR flight muscle ratio

IF induced flight

NF no flight

ROS reactive oxidative species

VF voluntary flight

WBF wingbeat frequency

CHAPTER I

INTRODUCTION

Life history theory is defined as a set of strategies, ranging from the cellular to the morphological level and includes the way an organism functions and behaves, that affect reproduction and longevity (Ricklefs and Wikelski, 2002). One fundamental issue in life history theory is how an organism's past behavior affects its present and future physiological performance. For example, vigorous exercise training in humans and other mammals has been shown to increase antioxidant capacity (Gündüz et al., 2004; Kayani et al., 2008), resistance to cellular oxidative damage (Radák et al., 1999), cognitive capacity (Suominen-Troyer et al., 1986), locomotor performance (Skalicky et al., 1996), motor coordination (Dorner et al., 1997), cardiac function (Jin et al., 2000), immune function (Utsuyama et al 1996), and lifespan (Paffenbarger et al., 1993; Lee and Paffenbarger, 2000). Alternatively, prolonged sedentarism in otherwise active mammal species can decrease running capacity (Swallow et al., 1998), lower aerobic capacity (Overton et al., 1986; MacNeil and Hoffman-Goetz, 1993), increase body mass (Mlekusch et al., 1996; Swallow et al., 1998), and decrease lifespan (Goodrick, 1980; Holloszy, 1988; Franco et al., 2005; Bronikowski et al., 2006).

The effects of physical activity (or lack thereof) are diverse, and the genetic underpinnings of them have been studied in several models. For example, Bronikowski et al. (2003) found that frequently-exercised mice had significantly fewer age-based changes in gene expression when compared to a sedentary group. The genes affected by physical activity included those responsible for inflammatory response (e.g. *LR8*, *SOCS*-3, etc), stress response (e.g. *MnK*, *GPx*, etc), signal transduction (e.g. *OSF*-2, *SLAP*, etc) and energy metabolism (e.g. *LONP* and *P14P5K*). Vigorous exercise causes rats to upregulate *PGC-1α* expression, which

increases mitochondrial biogenesis, putatively leading to higher aerobic capacity (Goto et al., 2000). Exercise training in rats also increases the concentration of proteins associated with neurogenesis (*N*-methyl-D-aspartate receptors and brain-derived neurotrophic factor) in the hippocampus (Farmer et al., 2004). The expression of genes related to synaptic plasticity, neuronal structure, and signal transduction also increase in exercised rats compared to sedentary rats (Tong et al., 2001). Exercise in humans increases expression of the angiogenesis gene *VEGF*, which contributes to an increased capillary density and higher oxygen transport across tissues to improve exercise capacity (Richardson et al., 1999). Exercise also elevates the expression of α-myosin heavy chain in the heart, a response thought to be associated with increased cardiac index and stroke volume index (Jin et al., 2000). When comparing more active mice to less active (but not sedentary) mice, Bronikowski et al. (2002) found that more active mice express lower amounts of the antioxidant enzyme catalase during exercise compared to the less active group. Lower antioxidant expression is hypothesized to correspond with lower production of reactive oxidative species (ROS) (Bronikowski et al. 2002). Reactive oxidative species (e.g. H_2O_2, $O_2^{\bullet -}$, and $OH^{\bullet}$) are created as byproducts of normal aerobic respiration and are known to cause damage to DNA, lipids, and proteins (Ames et al., 1993). Therefore lower levels of ROS production may echo the beneficial effects of exercise in vertebrates. Exercise in rats decreases the expression of mitochondrial proteins (e.g. NAD(P)H- oxidoreductase and cytochrome oxidase), possibly reflecting reduced turnover and replenishment of ROS-damaged proteins (Tong et al., 2001). However, the expression of various genes (such as *HO-1* and *UCP3*) that are hypothesized to minimize ROS damage increase during recovery from exercise, with expression peaking at about one to two hours after exercising (Pilegaard et al., 2000).

Far less is known about the effects of lifetime behavior patterns on physiological performance in invertebrates, the most speciose and abundant of which – the flying insects – lead much more aerobically variable and active lives than nearly all vertebrates due to their small size, poikilothermy, and extremely high energy costs associated with flight. For example, hovering insects have mass-specific metabolic rates that are many times higher than those of vertebrates and invertebrates (See review by Harrison and Roberts, 2000). Research to date suggests that flight activity (and suppression of it) in insects has few long-term parallels with cardiovascular/musculoskeletal exercise and sedentarism in vertebrates. Experimental, non-invasive suppression of flight behavior in certain dipterans (e.g. *Drosophila melanogaster*, *Musca domestica*) extends lifespan (Sohal and Buchan, 1981; Sohal et al., 1993; Agarwal and Sohal, 1994; Yan and Sohal, 2000; Magwere et al., 2006). In these studies, prohibiting flight behavior, either by wing removal or by housing organisms in an environment that prohibits flight, extends lifespan by decreasing the amount of oxygen consumed, which resulted in the reduced accumulation of ROS. Prohibiting flight behavior in *M. domestica* has been shown to slow the accrual of 8-hydroxydeoxyguanosine (8-OHdG) in nuclear and mitochondrial DNA (Agarwal and Sohal, 1994), and formation of 8-OHdG is due to oxidative damage to the DNA base guanine, which results in mutations in the DNA where adenine pares with 8-OHdG along the DNA strand (Michaels et al., 1992). Magwere et al. (2006) found that limiting flight behavior in *D. melanogaster* extended lifespan and accredited this to a decrease in the oxidative damage to lipids, as flight restriction lowered levels of polyunsaturated fatty acids and peroxidizability index. This means that flying makes flies more vulnerable to oxidative damage than not flying, and lipid peroxidation causes damage to the cell membrane and its end-products may cause damage to DNA in the form of DNA adducts (Burcham, 1998). In addition to lipid

peroxidation, protein carbonylation has also been found to be lower in flies prohibited from flying and is another potential mechanism by how limiting flight behavior increases longevity (Sohal et al., 1993; Yan and Sohal, 2000). Protein carbonylation occurs when an oxidative species reacts with the amino-acid side chain of proline, arginine, lysine, or threonine, and results in the alteration of the protein's form making it more prone to degredation (Nyström, 2005). Suppression of flight behavior also elevates levels of the antioxidant enzymes aconitase and adenine nucleotide translocase (Yan and Sohal, 2000), which helps maintain the functional activity of these proteins and delay the accumulation of oxidative damage inside the cell. A decrease in adenine nucleotide translocase function has been shown to lower the amount of ADP in mitochondria, thereby obstructing ATP formation (Yan and Sohal, 2000), as well as increase the formation of mitochondrial H_2O_2, which further intensifies oxidative damage to various cellular components (Esposito et al., 1999). Similarly, a decrease in aconitase activity reduces the amount of NADH and $FADH_2$ by interrupting glycolysis and the citric acid cycle, which ultimately suppresses cellular respiration activity (Ferguson et al., 2005).

Similar flight-related patterns of longevity and cellular stress phenomena are seen among behavioral castes of honey bees, *Apis mellifera*. In this species, workers perform few behaviors requiring flight in the early (or nursing) stages of their adult lives, and later transition to behaviors that increasingly involve flight, culminating in numerous daily, long-distance foraging flights. Early onset (i.e., precocious) foraging or extended daily flight duration while foraging decrease lifespan in honey bee workers (Neukirch, 1982), and certain antioxidant proteins are upregulated in foragers compared to nurse bees (Schippers et al., 2006; Wolschin and Amdam, 2007). Likewise, levels of flight muscle phosphofructokinase and cytochrome c oxidase decrease in older foragers (Schippers et al., 2006; Schippers et al., 2010), as does flight kinematics (Vance

et al., 2009). Young (8-10 day old) foragers diurnally upregulate antioxidant and heat-shock proteins in flight muscle in greater amounts than age-matched nurse bees, but this response largely disappears in old (30-32 day old) bees of either group (Williams et al., 2008). As discussed above, higher antioxidant expression may indicate higher levels of ROS production leading to deleterious effects inside the cell. In bumblebees, the suppression of foraging activity slightly increases body mass and the immune response later in life (Doums and Schmid-Hempel, 2000; Skandalis and Darveau, 2012), suggesting trade-offs between flight and other energy-demanding traits over the lifetime of a forager. Therefore, investment in flight behavior results in reduced energy allocation towards other life-history traits, such as immune function.

While flight behaviors decrease longevity and impose stress at the cellular level (e.g. oxidative stressors, lipid peroxidation, protein carbonylation), very little is known about the effects of sedentarism in insects. As described above, suppression of flight behavior extends lifespan and slows the accumulation of oxidative damage within flight muscles. However, there are few reported examples of muscle or neurological disuse pathologies in insects. Experimental wing removal in crickets accelerates weight and protein loss within the dorso-longitudinal flight muscle (Tanaka 1991; Gomi et al., 1995), while wing removal in newly-eclosed tsetse flies causes an increase in the sarcoplasmic fraction and a decrease in the mitochondrial and myofibrillar fractions of the dorso-longitudinal flight muscle as the flies age (Anderson and Finlayson, 1976). When *D. melanogaster* are prevented from flying during the first 5 days post-eclosion, steering control during flight is less precise, but maximal flight performance is not significantly different from flies permitted to fly beginning at eclosion, suggesting that the steering impairment is not due to a muscle disuse phenomenon, but instead a lack of neurological "practice" of fine control of aerial performance (Hesselberg and Lehmann, 2009).

Age based, functional senescence of flight activity has been well studied in *D. melanogaster* and other insects. In the Oregon R strain *D. melanogaster,* flight performance and wingbeat frequency (WBF) decrease after 28 and 42 days of age, respectively (Miller at al., 2008). Exploratory activity (distance travelled away from a central location per unit time), the proportion of time spent flying and negative geotaxis also decrease with age in *D. melanogaster* (Le Bourg and Minois, 1999; Gargano et al., 2005; Magwere et al., 2006; Simon et al., 2006). Wing kinematics and the frequency of foraging trips decrease in old honey bee foragers, although age has no effect on the flight performance of nurse bees (Tofilski, 2000; Vance et al., 2009). The resting metabolic rate of *D. melanogaster* also declines with age over the first two weeks of adulthood and then stays relatively constant throughout lifespan (Van Voorhies et al., 2004; Khazaeli et al., 2005). No study known to date has examined the change in flying metabolic rate over time in flying insects. The decline in flight performance in insects may be due to numerous molecular, cellular and morphological impairments associated with age. The flight muscles lose antioxidant capacity and accrue oxidative damage to lipids, proteins and DNA (Yan and Sohal, 2000; Magwere et al., 2006; Seehuus et al., 2006; Williams et al., 2008), and such damage possibly underlies the age-based impairment of glycolytic and electron transport chain enzymes, as described above, (Schippers et al., 2006; Schippers et al., 2010) and the ultrastructural degeneration of flight muscle mitochondria and sarcomeres (Sacktor and Shmida, 1972; Fernandez-Winckler and Cruz-Lamdim, 2008; Miller et al., 2008).

In this study, we investigated how lifetime patterns of flight behavior affect the age-based trajectory of flight performance in *D. melanogaster*. As described above, there are significant effects of varying flight behavior on dipteran flight muscle composition, control, and muscle oxidative damage, and we hypothesized that these effects, resulting from either disuse or overuse

of the flight muscles, would alter the flight performance of flies as they age. Specifically, we predicted that both the experimental suppression and elevation of flight behavior would, relative to control flies, lead to an early-onset reduction of relative flight muscle mass, flight ability and metabolism. To test these predictions, we separated 5 day-old female fruit flies into three life-long flight behavior groups: (A) those not allowed to fly (no flight or NF), (B) those allowed – but not forced – to fly (voluntary flight or VF), and (C) those mechanically stimulated to fly (induced flight or IF). For each group we measured flight capacity in a hypodense gas mixture (see Roberts et al., 2004), WBF, fresh body mass, and relative thorax mass (as a proxy for relative flight muscle ratio, or FMR) at 15, 35, and 65 days of age.

CHAPTER II

METHODS

Fly Culture and Age Cohorts

Colonies of *D. melanogaster* used in all experiments were derived from wild-caught flies captured near Houston, TX, USA, and maintained as an outbred population in captivity since 2008. The fly stock was maintained on standard *Drosophila* medium (Standard Medium, Indiana University Stock Center, Bloomington, IN, USA) in a climate-controlled incubator at 25°C under a 14 h: 10 h L:D photoperiod. In order to obtain age-matched cohorts of flies, females from the stock colony were permitted to lay eggs on a grape-agar laying medium, from which 1st instar larvae were collected 24 hours later and placed into standard food vials at a density of 100 larvae per vial. Adult flies were collected from these rearing vials at 24-hour intervals and used to populate the experimental treatment groups with age-matched (to within 24 hours) flies.

Lifetime Behavior Treatment Groups

Three different treatment groups were created to generate a broad range of daily flight activity. In all of the treatment groups, 5 day-old past eclosion female flies were segregated by sex into 0.24 L bottles at a density of 200 flies per bottle and maintained thereafter at 22 ± 1 °C. The lid of each bottle consisted of an inverted food dish that was changed daily. In the no flight (NF) group, nylon mesh was placed in the bottles to prevent the flies from flying but still allow walking and access to food. Flies in the voluntary flight (VF) group were kept in bottles without mesh. Flies in the induced flight (IF) group were also kept in bottles without mesh, but the bottles were adhered via velcro to a horizontal cardboard deck attached to the head of a vortex-type shaker. The shaker was programmed to gently shake, during the 14 hr light photoperiod,

every 5-10 min for 0.3 sec using an SLC programmable timer (Allen–Bradley, Milwaukee, WI, USA). The brief, abrupt movement of the bottle caused the flies to fly *en masse* approximately 120 times per day over the 14 hr light period. Preliminary control experiments were conducted with flies housed in bottles containing mesh and similarly agitated to assess the effect of shaking independent of increased flight frequency. The flight capacity and egg-laying rate of these flies did not differ from the NF group, indicating that the intermittent shaking alone did not affect flies. Female flies were sampled from the treatment groups at ages 15, 35 and 65 days for measurements of flight performance, metabolic rate and body mass.

Flight Performance Assay

Individual flies were sampled from treatment groups and screened for their ability to fly in a low density gas mixture of 21% O_2, 39.5% N_2, 39.5% He (density 0.81 g l^{-1}) at 22 ± 1 °C. Due to the greater power requirements of flying in a low density gas, this approach effectively reveals variation in insect flight performance (Roberts et al., 2004; Vance et al., 2009). Specifically, individual flies were placed into a 1.9 L covered plastic flight chamber that was perfused with the hypodense gas at a rate of 500 ml min^{-1}. The gas was mixed and regulated using a Sable Systems MFC-4 (Sable Systems Inc, Las Vegas, NV, USA). Flight performance was scored according to Frazier et al. (2008). Flies that showed normal, upward take-off flight behavior and could fly the full width of the chamber (12 cm) were categorized as performing a 'flight'; those that flew >5 cm but <12 cm, conventionally a take-off followed by an arching loop ending on the chamber bottom or a controlled decent off the chamber wall, were categorized as performing 'lift' (these flies were unable to maintain flight, but were considered different from 'no flight' because they could travel farther than the maximum jumping distance observed); and flies that traveled <5 cm and displayed an uncontrolled descent behavior were classified as

performing ‘no flight’. We tested a fly until it performed a flight or 3 min had passed. Flight performance was determined for 27-30 flies for each age x treatment group combination. An optical tachometer was used to measure wingbeat frequency (WBF) of flies performing ‘flight’ in the flight performance assay (Frazier et al., 2008). Additional flies were tested as needed to obtain WBF measurements of 12 flies for each age x treatment group combination. The tachometer recordings were then digitized and visualized using the RAVEN sound analysis program (Cornell Univ., Ithaca, NY, USA). Each recorded sequence contained 5–15 continuous wing beats. For each fly, WBF was determined to the nearest 0.2 Hz by dividing the number of clearly distinguishable, uninterrupted wing beats in the sequence by the duration of the sequence (measured to the nearest 0.001 s) (Frazier et al., 2008).

Body Mass and Metabolic Rate

Immediately after the flight assay, the live fly was weighed to the nearest 0.01 mg on a Sartorius BP211D balance (Sartorius Corporation, Edgewood, NY, USA). The fly was then frozen and its wings were removed. We estimated flight muscle ratio (FMR) as the ratio of dry thorax mass to dry body mass. The head, thorax and abdomen were separated and dried for 24h at 55°C, then immediately weighed using an Orion Cahn C-35 microbalance (±1 µg; Thermo Electron Corp., Beverly, MA, USA). The thorax primarily houses flight muscle and thus provides an index of flight muscle mass (Frazier et al., 2008).

Carbon dioxide production was measured using flow-through respirometry, in which a single trial consisted of 10 flies from a given age x treatment group combination placed together in a 30ml glass chamber maintained at 22 ± 1°C. Six trials using different flies were run for each age x treatment group combination. Dry CO_2-free air was passed through the chamber at 50 ml min^{-1}, controlled by a Tylan FC-260 mass-flow valve and a MFC-4 controller (Sable Systems

International, Las Vegas, NV, USA). Water vapor produced in the chamber was scrubbed from the air stream with a magnesium perchlorate drying column, and carbon dioxide was quantified in the excurrent air with a CA-10 analyzer (Sable Systems International, Las Vegas, NV, USA). Included in each run was a 2 min measurement of the chamber without any flies to establish baseline. Flies were placed in the chamber and, after an eight minute resting period, the chamber was tapped at 2 hz for approximately five minutes to stimulate them to fly as continuously as possible. Data were acquired by a UI-2 interface and analyzed with Expedata software (Sable Systems International, Las Vegas, NV, USA). Immediately after each metabolic trial, flies from that trial were weighed to the nearest 0.01 mg. The highest average age x treatment group metabolic rates obtained using this method were similar to metabolic rates of individual, continuously flying *D. melanogaster* measured by others (Wigglesworth, 1949; Lehmann et al., 2000; Lehmann and Schützner, 2010).

Statistical Analysis

To assess the effects of age and lifetime flight behavior on flight performance an ordinal logistic regression was conducted because the flight performance data was a categorical response variable. We also included interaction terms in the model to test the effects lifetime flight behavior had on senescence. We used a two-way Analysis of Variance test to analyze WBF, body mass, FMR, and metabolic rate in response to treatment and age. The normality of the data was tested with an Anderson-Darling test. *Post hoc* analyses were conducted using Tukey's test to determine which means were significantly different from one another. Type I error was set at 0.05.

CHAPTER III

RESULTS

Flight Capacity

Flight capacity was affected by age and lifetime flight behavior, as well as an interaction between age and lifetime flight behavior (Table 1). Among 15 day-old flies, nearly all flies from the voluntary flight (VF) and induced flight (IF) groups could fly, but 30% of the no flight (NF) flies were flight-impaired (Fig. 1). At 35 days of age, only 10% of the VF flies were flight impaired, compared to 50% and 30% for the NF and IF groups, respectively. By 65 days of age, nearly all flies in the NF and IF groups were flight-impaired, although 30% of the VF flies were still capable of flight.

Table 1. Ordinal logistic regression analysis evaluating the effects of age and treatment and their interaction on flight capacity in *D. melanogaster.*

Variable (d.f.)	Coefficient	s.e.m.	*P*-value	Odds ratio
Age (2)	-3.5036	0.5498	0.0000	0.0301
Treatment (2)	-1.9023	0.4772	0.0001	0.1492
Age x Treatment (4)	1.0886	0.3379	0.0013	2.9702

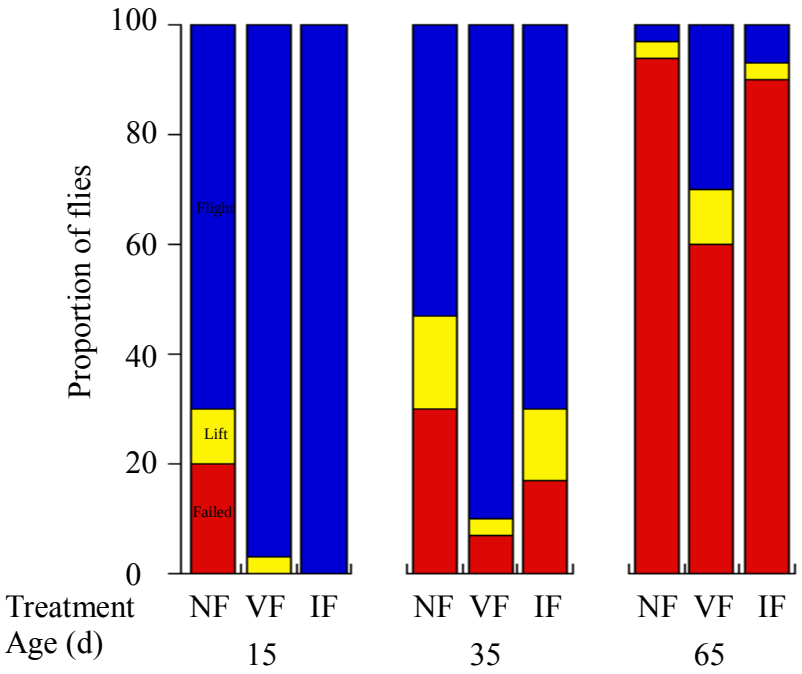

Figure 1. Flight capacity as a function of age and lifetime flight behavior in *D. melanogaster*. Blue indicates proportion of flies that could fly normally, yellow indicates generation of lift (limited flight), and red indicates failed or no flight (see Methods section for details). See text for label descriptions. N = 27-30 flies for each age x treatment group combination.

Body Mass, Flight Muscle Ratio, Wingbeat Frequency and Metabolic Rate

There was an effect of age on body mass, as well as an interaction between age and lifetime flight behavior (Table 2A). At 15 days of age, NF and VF flies were slightly (10%) heavier than IF flies, but body mass was not significantly different among lifetime behavior groups at 35 and 65 days of age. Except for the slightly heavier flies in the 15 day-old NF and VF flies, body mass was generally constant at 1.05-1.12 mg across all ages (Fig. 2). Flight muscle ratio (FMR) was affected by age and lifetime flight behavior, and there was an interaction between age and lifetime flight behavior (Table 2B). At 15 days of age, IF flies had a slightly higher FMR than NF and VF flies (Fig. 3). The FMR of IF flies remained constant with

age at about 41%, while the FMR of NF flies was about 37% at all ages. The FMR of the VF flies increased from 37% to 40% over the duration of the experiment.

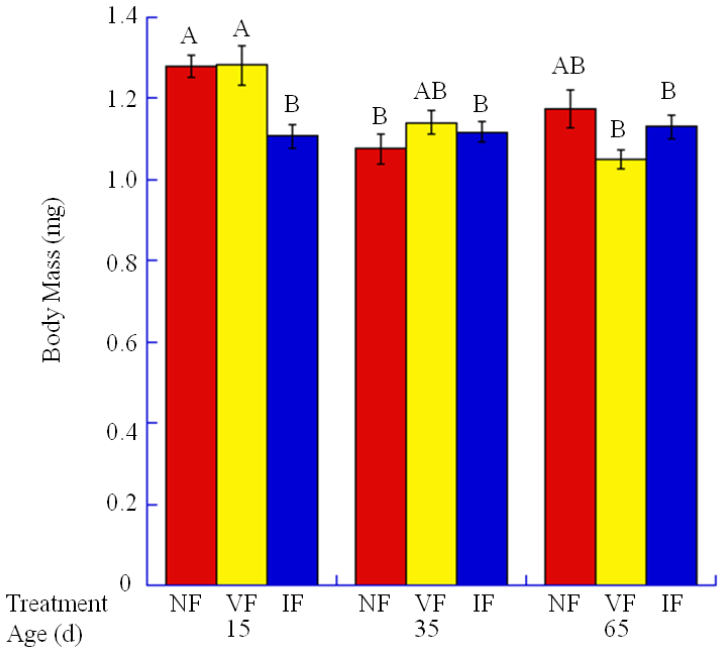

Figure 2. Body mass as a function of age and lifetime flight behavior in *D. melanogaster*. Means that do not share a letter are significantly different. See text for label descriptions. Error bars represent s.e.m. N = 27-30 flies for each age x treatment group combination.

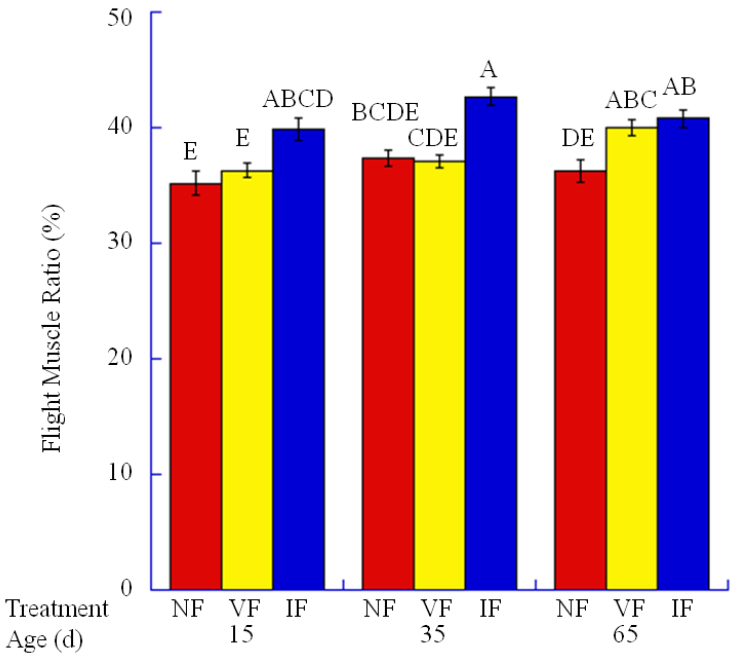

Figure 3. Flight muscle ratio as a function of age and lifetime flight behavior in *D. melanogaster*. Means that do not share a letter are significantly different. See text for label descriptions. Error bars represent s.e.m. N = 27-30 flies for each age x treatment group combination.

There was an effect of age on wingbeat frequency (WBF), as well as an interaction between age and lifetime flight behavior (Table 2C). In the NF group, WBF was highest at 35 days of age and not significantly different from the highest WBFs of the VF and IF, which occurred at 15 days of age (Fig. 4). In the VF group, WBF decreased between 15 and 35 days of age, but did not significantly further decrease at 65 days of age. In the IF group, WBF decreased between 15 and 35 days of age and between 35 and 65 days of age, but only the decrease in the latter period was statistically significant.

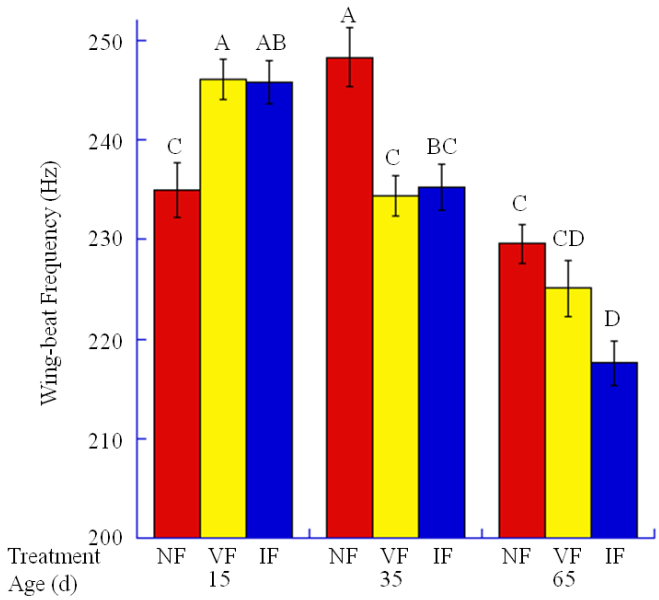

Figure 4. Wing-beat frequency as a function of age and lifetime flight behavior in *D. melanogaster*. Means that do not share a letter are significantly different. See text for label descriptions. Error bars represent s.e.m. N = 12 flies for each age x treatment group combination.

Metabolic rate was affected by age and lifetime flight behavior, and there was an interaction between the age and lifetime flight behavior (Table 2D). In the NF group, metabolic rate was low and unaffected by age (Fig. 5). In the VF and IF groups, metabolic rates were highest at 15 days of age and dropped as the flies aged. Metabolic rates of flies in the VF group fell by 35 days of age and again by 65 days of age. In the IF group, metabolic rate dropped dramatically (57%) between 15 and 35 days of age, but did not further drop between 35 and 65 days of age.

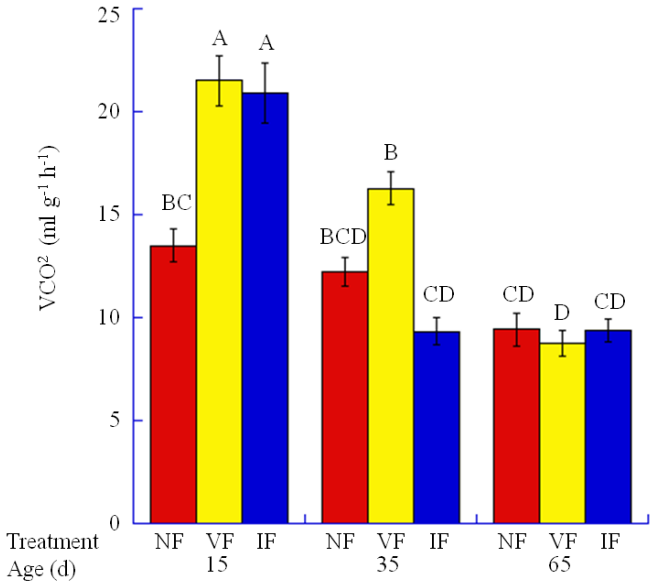

Figure 5. Metabolic rate during "agitated" flight as a function of age and lifetime flight behavior in *D. melanogaster*. Means that do not share a letter are significantly different. See text for label descriptions. Error bars represent s.e.m. N = 6 for each age x treatment group combination.

Table 2. ANOVA tables for factors affecting: (A) body mass, (B) flight muscle ratio, (C) wing-beat frequency, and (D) metabolic rate in *D. melanogaster*.

	Variable (d.f.)	F-value	*P*-value
A. Body mass (mg)			
	Age (2)	10.64	<0.001
	Treatment (2)	2.47	0.087
	Age X Treatment (4)	5.62	<0.001
B. Flight muscle ratio (% body mass)			
	Age (2)	5.63	0.004
	Treatment (2)	27.23	<0.001
	Age X Treatment (4)	2.58	0.038
C. Wing-beat frequency (Hz)			
	Age (2)	49.55	<0.001
	Treatment (2)	2.90	0.060
	Age X Treatment (4)	10.58	<0.001
D. Metabolic rate (ml g^{-1} h^{-1})			
	Age (2)	86.95	<0.001
	Treatment (2)	13.86	<0.001
	Age X Treatment (4)	13.36	<0.001

CHAPTER IV

DISCUSSION

The senescence of flight ability in aged insects is well-established (Le Bourg and Minois, 1999; Magwere et al., 2006; Simon et al., 2006; Miller et al., 2008), and the results of this study expand the understanding of this process by demonstrating that variation in lifetime flight behavior, in addition to age, affects the onset and magnitude of flight senescence. Elevated flight frequency in the induced flight (IF) group hastened the onset of flight senescence compared to the voluntary flight (VF) group, and the pace of flight senescence in the VF and IF flies between 35 and 65 days of age was the most rapid over any time period in the study. The accelerated pace of flight senescence in older VF and IF flies may have been due to a more rapid accrual of molecular, cellular and/or internal morphological damage in the flight apparatus (although wing-wear, while not specifically measured, was not noticeable in the study). Suppression of flight behavior in dipterans slows the accumulation of cellular oxidative damage in flight muscles and increases lifespan (Agarwal and Sohal, 1994; Yan and Sohal, 2000; Magwere et al., 2006), which might lead one to predict that flight ability in the NF group would persist later into life compared to the VF and IF groups. However, the no flight (NF) flies lost their flight ability earliest, indicating the presence of detrimental disuse phenomena that outweighed presumed beneficial effects of mitigated oxidative/ultrastructural damage.

Aging and Senescence of Flight Performance

Flight performance, wingbeat frequency (WBF) and metabolic rate decreased in older flies, and such trends are well-documented for *Drosophila* and other insects (Rockstein and Bhatnagar 1966; Le Bourg and Minois, 1999; Tofilski, 2000; Simon et al., 2006; Miller at al., 2008; Williams et al., 2008; Vance et al., 2009). The senescence of flight performance observed

in our study may be due to the loss of antioxidant capacity and accrual of oxidative damage. Decreases in antioxidant capacity (e.g. aconitase, adenine nucleotide translocase) and increases in the accumulation of oxidative stressors (e.g. 8-OHdG, glutathione, hydrogen peroxide, etc) with age (Yan and Sohal, 2000; Magwere et al., 2006; Seehuus et al., 2006; Williams et al., 2008) result in damage to DNA, membranes, and proteins (Beckman and Ames 1998). This damage is predicted to impair glycolytic and electron transport chain enzymes, which inhibits cellular respiration and results in lower oxygen consumption (Ferguson et al., 2005; Schippers et al., 2006; Schippers et al., 2010). This damage may also result in the degeneration of flight muscle ultrastructure (Sacktor and Shmida, 1972; Fernandez-Winckler and Cruz-Lamdim, 2008; Miller et al., 2008), which in turn might explain the lower flight performance and metabolic rates of aged flight-active flies in our study. Flight muscle mass decreases steadily with age in certain insects (Ready and Josephson, 1982; Stjernholm and Karlsson, 2008), although this effect did not occur in our study (Fig. 3). Wing wear is another aging phenomenon common to flying insects, especially large species (see Foster and Cartar, 2011), but this too was not observed in our study, nor has it been well documented in very small insects such as *Drosophila*. It is also possible that the variation in flight performance observed in our study is due to genetic variation between the treatment groups. Montooth et al. (2003) compared flight performance (by measuring flight velocity) in a recombinant inbred line of *D. melanogaster* and with the use of quantitative trait locus (QTL) mapping found that variation observed in flight performance between individuals was due substantially to a genetic component. Therefore it is possible that the differences observed in the present study is due to genetics as well as the treatment environment and QTL mapping should be conducted to help determine what role genetics played on the variation observed.

Previous Flight Activity and Senescence

Elevated levels of previous flight activity accelerated the onset of flight senescence. At early ages, flight performance did not differ between the VF and IF groups and were at peak levels. By mid-age, flight performance had dropped about twice as much in the IF group than in the VF group, and in old flies about 30% of VF flies could still fly (compared to 10% in the IF group). Variation in body mass and FMR played little if any role in the differences in flight senescence patterns between the IF and VF flies. Fresh body mass (more of which should impair flight – see Roberts et al., (2004)) was slightly, but significantly, higher in VF flies than in IF flies only at 15 days of age, and was the same in the two groups at older ages. Furthermore, FMR (which should aid flight performance) was slightly higher in IF flies than in VF flies at 15 and 35 days of age but was equivalent between these groups at 65 days of age. Wingbeat frequency is a primary determinant of aerodynamic power (Dudley 2000), and therefore is expected to be highest in flight-capable flies. WBF declined with age in the VF and IF groups between 15 and 35 days of age and continued to decline between 35 and 65 days of age, although no significant difference in WBF was observed between the two groups at any age. Thus WBF does not explain the differences observed in flight performance between older VF and IF flies. Similar to what was observed in WBF, metabolic rate decreased with age in both the VF and IF groups. However, metabolic rate decreased most rapidly in the IF group, reaching its minimum by 35 days of age. Metabolic rate in the VF group decreased slowly with age and reached its lowest point at 65 days of age, where it was equal to the measurements of the IF group. In the few other studies to measure the effects of age on insect flight performance and metabolism, Vance et al. (2009) and Miller et al. (2008) found that flight performance decreased with age in foraging

honey bees and *D. melanogaster*, respectively, while Schippers et al. (2010) found that the flight metabolic rate of honeybees did not change with age.

Suppression of flight behavior also led to a premature decrease in flight ability and failure to attain the peak metabolic rates exhibited by 15 day-old VF and IF flies. The NF flies did not attain the peak WBF of 15 day-old VF and IF flies (~247 Hz) until 35 days of age, at which time they were even more flight impaired than 15 day-old IF flies and 35 day-old VF and IF flies. This fact, along with the small variation in fresh body mass and FMR among ages and behavior groups, indicates that the premature loss of flight ability of NF flies was not due to major morphological or gross kinematic disadvantages (although a more complete aerodynamic analysis is necessary to confirm the latter). Instead, disuse phenomena limiting flight control and metabolic capacity could be the major factors underlying the impairment of flight ability in the NF group. Indeed, when the flight of *D. melanogaster* is suppressed during the first 5 days after eclosion, flight properties associated with steering control (e.g. turning rate, control of stroke asymmetry, etc.) in 5 day-old flies are impaired, although there is no effect on WBF and peak horizontal speed (Hesselberg and Lehmann, 2009). Using three treatment groups very similar to ours, Anderson and Finlayson (1976) showed that post-eclosion suppression of flight behaviors in tsetse flies causes an increase in the sarcoplasmic fraction and a decrease in the mitochondrial and myofibrillar fractions of flight muscle, while long-term increases in flight frequency (via daily tapping on the cage) induces opposite long-term effects. We are currently conducting experiments to determine if similar muscle composition phenomena, which could help to explain the observed patterns in metabolism, are occurring in our NF, VF and IF groups of *D. melanogaster*.

The results of other studies that have manipulated insect flight activity strongly suggest that flight behavior accelerates senescence and decreases longevity due to the production of reactive oxygen species and the accumulation of oxidative damage within cells. The strongest evidence for this argument is that the developmental or experimental suppression of flight behavior in flies and bees slows the senescence of antioxidant defenses and the accumulation of oxidative damage (Sohal and Buchan, 1981; Sohal et al., 1984; Agarwal and Sohal, 1994; Yan and Sohal, 2000; Magwere et al., 2006; Williams et al., 2008). However, no studies to date have tested whether experimental increases in flight behavior yield supra-normal accumulation of reactive oxidative species (ROS) and oxidative damage to cellular macromolecules. Our preliminary comparisons of longevity in the NF, VF and IF groups suggests this may be the case, as the longevity of VF flies is greater than IF flies but lower than NF flies, and aged IF flies have much higher levels of mitochondrial degradation than NF and VF flies (G. Mancinelli and E. Martinez, personal communication). However, in an interesting parallel to human exercise, experimental increases in the frequency of walking behavior in *D. melanogaster* slows aged-base decreases in walking mobility and cardiac function (Piazza et al., 2009), indicating that the type of locomotor behavior, perhaps related to the contraction frequency and relative mass of muscles involved, can have strong effects on health and senescence. The gene *spargel* (*srl*) in *D. melanogaster*, a homolog of *PGC-1α* found in vertebrates, has been found to increase mitochondria formation and activity, similar to the functions of *PGC-1α* in mice (Tiefenbock et al., 2010). Moreover, overexpression of *srl* in *D. melanogaster* increases walking mobility and higher cardiac function (Tinkerhess et al., 2012). These studies suggest that there are similarities between exercise, physiological performance, and gene expression in vertebrates and

invertebrates, however, this relationship has not been well studied and more research must be done to determine the extent of these similarities.

Conclusions

This is the first study to assess how age and the experimental suppression and enhancement of lifetime flight activity affect flight performance in a volant species. Elevated levels of flight activity hasten the senescence of flight performance metabolism in a pattern similar to the more rapid senescence of antioxidant defenses and accumulation of oxidative damage in flight-active *versus* flight-inactive insects. The loss of flight ability is likewise hastened by the lifetime suppression of flight behavior, but likely due instead to disuse phenomena that are not yet completely understood, but may include lack of neuromuscular exercise and changes in flight muscle composition. Research is ongoing in our lab to further determine how lifetime flight behavior, including alternating periods of activity and sedentarism, affects longevity and age-based patterns of flight performance, flight muscle ultrastructure, fuel substrate usage and energetic allocation strategies.

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