

*Bodybuilding Bumblebees: How exercise affects bumblebee (*Bombus impatiens*) flight muscle*

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## Abstract

Insect flight muscle is the most metabolically active tissue known, allowing for production of the large forces necessary for flight. Flight ability varies among insects and with insect age, which is partially due to differences in muscle. However, very little work has examined whether and how insect muscle may respond to exercise. One key response may be a change in the amount of flight muscle relative to body mass ("flight muscle ratio", FMR), analogous to bulkier muscle in bodybuilders. We examined differences in FMR among bumblebee (*Bombus impatiens*) sisters differing in body size and in "training". We trained the bees by periodically having them lift beaded strings during flight. This approach allows direct observation of how many weights are being lifted (their performance), and ultimately, provides a starting point for determining how exercise may affect insect flight muscle. Aside from changes in FMR, bees that regularly exercise may have elevated mitochondrial density and increased enzymatic activity.

## Why should we test for exercise?

Research on the exercise habits of animals is relatively unexplored, but it may tell us some interesting things about how animals maintain metabolically demanding muscle tissues. Energetics is the field of science dedicated to studying how organisms use energy and most studies only focus on the tradeoff between overall physical fitness and reproductive ability in the long-term. Little research has tested whether repeated short bursts of energy have an impact on long-term success. A reason for this is that these short bursts behaviors do not fully exhaust energy stores nor are they simple to measure. Because research in animal exercise is new, there are few concepts guiding its development as a topic of science. In his review, Halsey (2016) gives general concepts that he believes provide a good framework for experiments to test for exercise. These concepts support why we think animals may exercise and why *Bombus impatiens* bumblebees are the perfect model organism for this research with their large flight muscles.

The first concept that Halsey (2016) proposes is that an organism which is subject to quick muscle fatigue would be at a disadvantage. If short bursts of energy exhaust an animal too quickly, then it is difficult to escape from a predator or be competitive enough to acquire a mate. Secondly, there is evidence that muscles do change with exercise. Mice naturally are drawn to run on wheels and we see accompanying physiological changes in the muscle. This occurs both in the lab and in nature (Halsey, 2016). The third concept is that there is evidence that animals do responsively change their body condition to their environment. Harbor porpoises (*Phocoena phocoena*) alter their body condition so that they are more slender and better

able to escape predation from dolphins. The risk of predation is greater than the risk of starvation. The fourth concept is that some animals will need greater physical fitness than others based on their ecological role. The giant panda (*Ailuropoda melanoleuca*) is an animal that no longer has predators and would not benefit from physical fitness. Lastly, some animals can circumvent expending energy on physical fitness altogether because they have an endogenous mechanism which regulates muscle growth and efficiency. This is important because in organisms that we see changes in body condition, we may not know whether it is the result an endogenous mechanism or exercise. When we analyze *B. impatiens* using this conceptual framework, it is clear that researching whether they exercise to maintain flight muscle could tell us a lot about how they are able to perform such metabolically exhausting tasks that are key to their survival.

## **Why are bumblebees a good model for exercise research?**

Bumblebees are a good organism for exercise research because we can apply Halsey's (2016) five concepts. First, many species of bumblebees are capable of great flights which consist of long foraging trips and flights at altitudes higher than Mount Everest (Dillon & Dudley, 2014), which leads us to assume that their muscles do not fatigue quickly. Second, they carry large loads regularly meaning that we could potentially see a change in muscle because of increased load lifting. Third, we think bumblebees may have the ability to change their body condition if forages are further from the nest or if predation is high. Fourth, their ecological role as pollinators and foragers suggests that they would benefit from exercise. Lastly, we know that bumblebees do have changes in muscle as they age and testing for response to exercise

versus no exercise will help us determine if this is an endogenous mechanism. When these concepts are considered together, Halsey (2016) suggests the challenge in using them lies in constructing viable experiments.

Exercise experiments must present a physical challenge to an animal and allow us to observe an animal's behavior and activity levels in response to that challenge (Halsey, 2016). The way that we physically challenged our bumblebees was by making a beaded string and attaching it between their abdomen and thorax. This models resistance training and helps us assess performance. As the bumblebee flies higher, it lifts more beads; therefore, it lifts a greater weight. We can then analyze the muscle and see if there is a relationship between performance and muscle properties.

## **How does flight muscle work and what can we measure?**

Flight muscle ratio (FMR) is the mass of tissues dedicated to flight muscle in comparison to the total body mass. James Marden (1987) found it to be a good indication of how much lift an animal can maximally produce based on its body mass. Lift is the force needed to overcome gravity and in still air, the flight muscles completely produce it. This is known as powered-lift. Powered-lift can be measured unweighted, but weighted powered-lift provides an indication of how much lift these animals can maximally generate. Marden (1987) performed experiments testing the limits of maximum powered-lift by attaching lead weights to various species of flying animals. This is important to our experiment because it confirms that flight muscle ratio is a good property to measure and that we had a good method for weight lifting.

Marden's (1987) methods for experimentally increasing the load that the animal lifted were important in determining accurate maximum lift production. Researchers

attached weights to the abdomens of insects and to the lower back of bats using a mixture of beeswax and resin, and attached weights to birds by wrapping strips of lead around the birds' legs. When a weight was not enough to prevent takeoff, researchers attached a heavier weight. They determined maximum lift by using the force-equivalent in Newtons, which was the mass of the animal plus a measurement halfway between the maximum mass lifted and the minimum mass that the animal could not lift (Marden, 1987). While they could attach weights to most of the animals successfully, it was challenging to attach weights to insects without disrupting the center of mass.

Scientists wanted to add weights close to the center of mass of animals to limit any irregular flight patterns caused by an uneven distribution of weight, but they also were interested to know how much placement of weight would affect mass-specific weight-lifting. The group of insects that made this easiest to test were dragonflies since they have longer thoraces. They attached weights on some dragonflies to the anterior abdomen, which is posterior to their center of mass; and others on the extreme posterior end- very far from the center of mass. They found that there was not a significant difference in maximum lift production (Marden, 1987), but to simulate the most natural increase in load-lifting for *B. impatiens*, we wanted to be as close to the center of mass as possible. Although maximum load-lifting may be the same despite the attachment, there may be unnecessary changes in kinematics which we want to limit. Kinematics are measurements of how the wing moves during flight. The center of mass is located at the abdomen and close to the honey stomach for *Bombus impatiens* (Mountcastle et al., 2016) and our weight-lifting method maintains an increase in load-lifting as close to this region as possible.

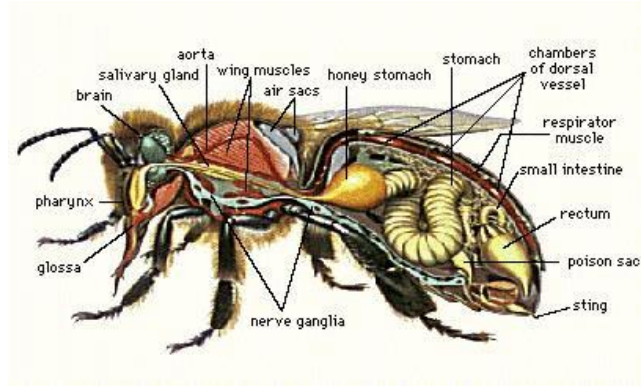


Figure 1: Bumblebee internal anatomy. We know the center of mass to be close to the honey stomach. Notice how the honey stomach (crop) is very close to the separation between the thorax and the abdomen (Bees-online, 2017).

There were significant results for the relationship between flight muscle ratio and lift per unit body mass for conventional wingbeat flyers- just like bumblebees. FMR for conventional fliers and mass-specific body mass had a correlation coefficient of .919 meaning they are likely closely related and an  $r^2$  value of .845 meaning that flight muscle ratio could explain 84.5% of the variance in the maximum-lift force relative to body mass (Marden, 1987). This means that FMR should tell us how much an animal is able to lift relative to its body mass, but FMR alone does not give an indication of how much an animal can maximally lift. This is helpful in studying bumblebee flight muscle because it tells us that FMR is a good measure to compare to overall performance.

It is required that only 12 to 16% of a flying insect's body mass be dedicated to flight muscle to achieve flight (Marden, 2000), but we want to know how aerial competitive ability is affected by having a lower FMR. *Plathemis lydia* dragonfly males are known to have the one of the highest FMRs of any flying animal at 0.60 (Marden, 1989), but prior to this research, it was unclear as to what advantage that may have. Marden (1989) experimentally decreased the FMR of these dragonflies by tying weight belts around their abdomen. This increased the overall mass dedicated to non-muscle tissues, thereby decreasing FMR. Researchers observed that the males who had weight

belts had poorer mating success, as they competed in aerial contests less and expended less energy (Marden, 1989). Therefore, we have evidence that a higher FMR is advantageous in being more successful in specialized flights. While this research was on dragonflies, we may see a similar competitive advantage with a higher FMR in worker bumblebees despite some differences in muscle structure.

Dragonflies and bumblebees have different flight muscle mechanisms. Flight muscles in dragonflies are the primitive mechanism while those in bumblebees are the advanced mechanism. The differences are in the muscle attachments and amount of neural activation necessary.

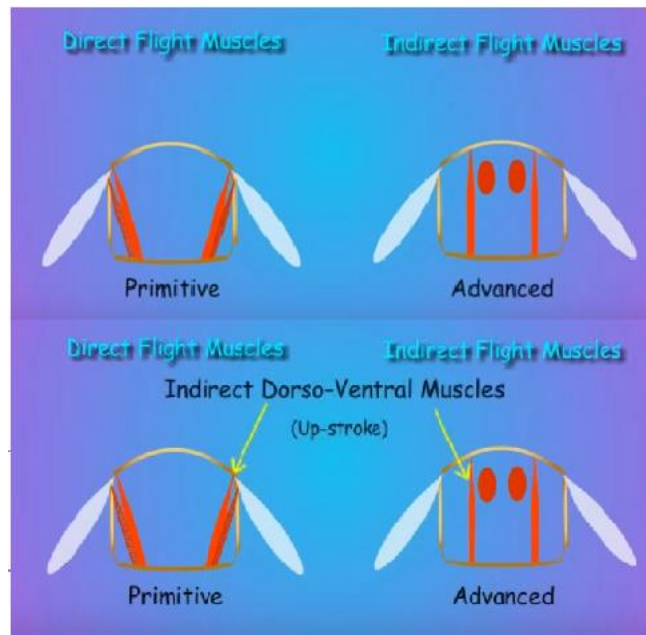


Figure 2: From Keeley (2014b). Here we see the two different types of insect flight mechanisms. Dragonflies still have an indirect Dorso-Ventral Muscle to produce the up-stroke, but the down stroke is produced by the direct muscle attached to the wing.

The primitive mechanism consists of an indirect dorso-ventral muscle attached to the cuticle that produces the up-stroke, and a direct muscle attached to the wing which produces the down stroke (Keeley, 2014b). Bumblebees and most modern insects have two indirect muscles that produce both the up-stroke and the down stroke (Keeley,



2014a). Direct muscles are also known as synchronous and indirect muscles are known as asynchronous. Synchronous muscles have a 1:1 neural activation scheme that is typical of muscle contraction- calcium is released, contraction is initiated and then the sarcoplasmic reticulum uptakes the calcium to stop contraction. Advanced insects have smaller synchronous muscles that do not produce a large amount of power, but do respond quickly and are used to facilitate this oscillating asynchronous contraction. Asynchronous muscles require less neural activation of the muscles per contraction. It takes about one action potential for every ten contractions. This makes these muscles optimal for functioning at high frequencies. The reason that synchronous muscle is less able to accommodate for high frequencies is that they have a higher density of sarcoplasmic reticulum and narrow myofibrils. While this does allow for greater pumping of calcium and calcium cycling with a shorter response time in activation and deactivation, more sarcoplasmic reticulum means less space for other important tissues such as force generating muscle cells and energy-providing mitochondria (Marden, 2000). Although these mechanisms of flight muscle usage are different, research on invertebrate flight with dragonflies still has relevance in testing properties like FMR in bumblebees.

Innervation of *B. impatiens* flight muscle is also important as its specificity helps us better understand the mechanism of contraction and deformation of the cuticle. A key characteristic of the thorax of advanced, asynchronous flight muscle is that a great deal of innervation occurs at the dorso-ventral muscle rather than the longitudinal muscle. Four nerve branches are identified, but only one branch appears to innervate the longitudinal muscle (Ikeda & Boettiger 1965). We think this may be because the dorso-ventral muscle produces the up-stroke which appears to be a slightly less

energetic process than producing the down stroke which is more responsible for overcoming the forces of gravity. Having the down stroke produced by stretch activation is a more efficient use of neural activation.

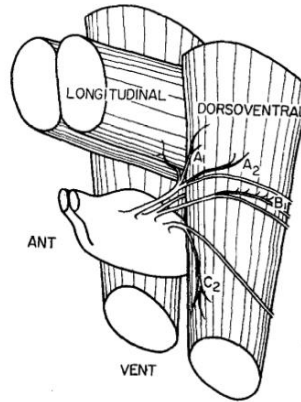


FIG. 1. Nerves leading from the second thoracic ganglion of *Bombus* to the longitudinal and dorsoventral flight muscles.

Figure 3: (Fig.1 Ikeda &Boettiger, 1965) Innervation of indirect flight muscles of *B. impatiens*.

Nerve impulse does not contract the indirect flight muscles, but instead activates them. This activation keeps the muscles in a constant state of excitation meaning that any stretch can cause contraction at a very high frequency. One set of the muscles develops tension and the other is stretched by that tension pulling the nodes of the thorax together. The longitudinal muscle develops tension first which causes the dorso-ventral muscle to stretch as the cuticle is pulled in. Once the longitudinal muscle has reach its equilibrium point, it immediately relaxes. This causes the down stroke. Relaxation of the longitudinal muscle stimulates an increase in tension in the dorso-ventral muscles. The muscle then snaps down and the wing is lifted. The importance of this system is that it allows wing beat frequency to be very high (Keeley, 2014a). We also must acknowledge the speed of this contraction is regulated at the molecular level.

The molecular components of insect flight muscle include a very fast actomyosin complex. As insect flight continued to evolve, the motor protein myosin II had to alter

its interactions with the actin filament because this interaction is primarily responsible for how fast a muscle will contract (Swank et al., 2006). A typical contraction is initiated from a neural message, as mentioned previously. This message causes calcium to bind to troponin, which causes tropomyosin to shift and expose the myosin binding site. These exposed myosin heads are then able to bind to the actin filaments. The myosin heads pivot and pull on the actin filament. Next, ATP binds to the myosin head which allows for release from actin and returns the myosin head to a state of readiness by hydrolyzing ATP. It then continues this process of attaching and pulling on the actin via this ATP hydrolysis. This is called the cross-bridging cycle and will continue to occur if calcium is still present (Fua, 2011). The mechanism found to be responsible for the rate-limiting step in slow muscle contractions is the release of ADP at the myosin head, but this did not appear to be true for faster muscle types. Insects with asynchronous flight have three key enzymatic adaptations that are important for speeding up this process. First, these asynchronous muscles have the highest rate of detachment of actin from myosin. Second, there is a weak affinity for ATP to myosin. Lastly, the rate limiting step was found to be facilitated by the inorganic phosphate release (Swank et al., 2006). Knowing the molecular details of insect flight muscle contraction is key to understanding why this tissue is so metabolically demanding.

Metabolic pathways for flight muscles are very important and highly evolved processes that are great parameters for measurement of activity. Scientists were interested in how these pathways may be allometrically scaled. Darveau (2005) analyzed orchid bees and how the evolution of metabolic rates was paralleled with flux capacities of hover flight. Flux capacity is just a measure of how much enzyme is active in the processes of substrate catabolism, the Krebs cycle, and the electron transport chain.

What they found was found that hexokinase activity scaled allometrically with an exponent that was very close to the exponents for wingbeat frequency and mass-specific metabolic rate (Darveau, 2005). Further research studies of allometrics attempted to explain why there was still so much variation in the metabolic rate of bumblebees. It was found that body size alone is only able to explain about 50% of the variation in flight muscle metabolic rate, and that many other factors should be acknowledged. They considered that those other factors may be related to wing morphology and flight muscle enzyme activity which still only explain about 75% of the variation (Skandalis & Darveau, 2012). The significance of knowing this is should we be interested in looking at specific enzymes, we know which ones will most closely explain what we see with kinematics and performance with mass-specificity in exercised and non-exercised bees.

### **Where do we start our exercise experiment?**

We had to synthesize this information and determine relevance in the short-term so that we could find a good starting point. We wanted to first develop a representative sample of flight muscle ratio across the colony to see if there was a trend. We gathered this data by pulling bees immediately from the colony, euthanizing them via the freezer method, squeezing out the crop content and then massing them. All masses were recorded in grams. Once the body mass was determined, we began dissecting the wings, head, abdomen, and legs from the thorax. We then massed the thorax with all the muscle contents, cut the thorax in half and used a basic 0.5 M NaOH solution to perform the digestion. This muscle digestion method was described by Marden (1987). Cutting the thorax in half allows the basic solution to penetrate far into the muscle, making for easier muscle removal. Digestion periods lasted 2-3 days and the cuticle was

dried approximately one hour post-removal of remaining muscle contents. The data for the colony is shown below.

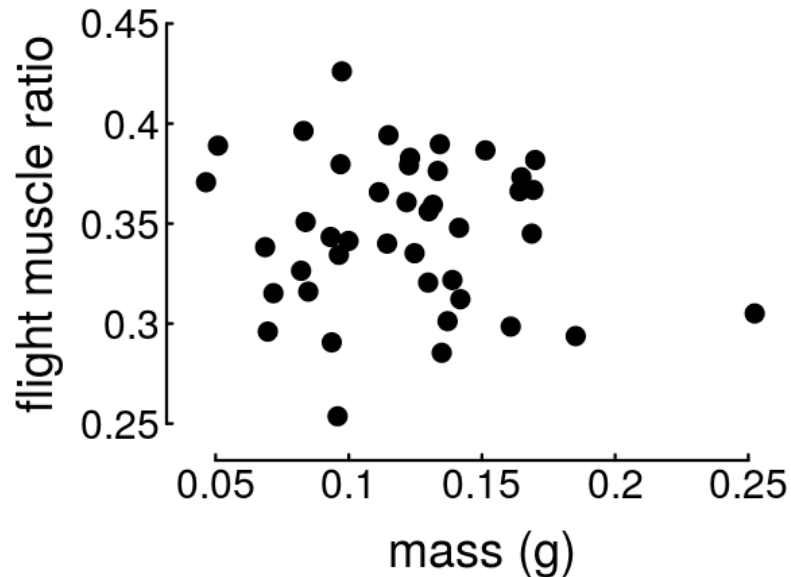


Figure 4: mass vs. flight muscle ratio. No significant relationship was determined.

We found no significant results between mass and flight muscle ratio. This could be the result of not controlling for all confounding variables, in particular- age of the bee- or it could mean that there was no relationship. If there is truly no relationship, this suggests that FMR may be related to other morphological variables. An interesting observation from performing these digestions was that we even saw variation within the thorax mass among *B. impatiens* of relatively the same total body mass.

We were still interested in testing flight muscle ratio with performance, but there was a very specific phenotype we were looking for in flight trial bees. We wanted workers because they are the foragers of the hive; they had to have good quality wings with very little wing wear; and had to be very active, so we could be confident that they would fly. The mere ability to fly was not enough to qualify as a successful flight.

Bumblebee flight is already a very dynamic process in terms of kinematics- especially since these insects typically fly through rough air in nature. In planning this experiment, we decided that hovering would be the only accepted flight pattern as this most mirrors how bumblebees fly in nature while pursuing forages or carrying loads. Another reason that hover flight was chosen was because it produces optimal wing kinematics that minimize energy usage (Berman & Wang, 2007).

Many attempts later, we found that no matter how closely the bumblebees fit our chosen phenotype, they did not want to fly for us. We initially thought it may have been because we were temporarily anaesthetizing them with a 30-minute cold treatment. The reason we did this was so we could tie the weight-lifting apparatus around them more efficiently. Even when we stopped anaesthetizing them, we did not see an increase in flight activity. Many reasons could explain why these bees did not want to fly. Overall, we think this may be a result of the bees not having the opportunity to forage. We wanted to keep them in the colony box to control for amount of flight they got, but we think it adversely affected our experiment. This is still relevant to our question of whether bees exercise or not. Are they not able to fly because they have not been foraging and have not developed their flight muscles, or is it because they have food easily accessible to them and they feel no need to fly and forage? Could it be that *B. impatiens*, a typical “lab bee” has been bred in captivity and lived in boxes for so long that, as a species, flight is no longer key to their survival? Although we had little success with getting bees to lift our beaded string, we were still able to get kinematics data from bees that we did allow the opportunity to forage.

We were still interested in flight muscle ratio and how it may relate to kinematics. The two parameters we were interested in were stroke amplitude and wingbeat

frequency. Stroke amplitude is the angle between the tip of the wing on the upstroke and the tip of the wing on the down stroke. Wingbeat frequency is how often the wing oscillates per second. Literature typically acknowledges that stroke amplitude is the kinematic adjustment bumblebees make to changing conditions, and that the wingbeat frequency of an individual remains the same regardless of the challenge. Footage was recorded using a high-speed camera at a rate of 2,500 frames per second and from that footage, kinematics data was extracted.



Figure 5: Stroke amplitude. This is the angle measured from the tip of the forewing on the up-stroke to the tip of the forewing on the down stroke.

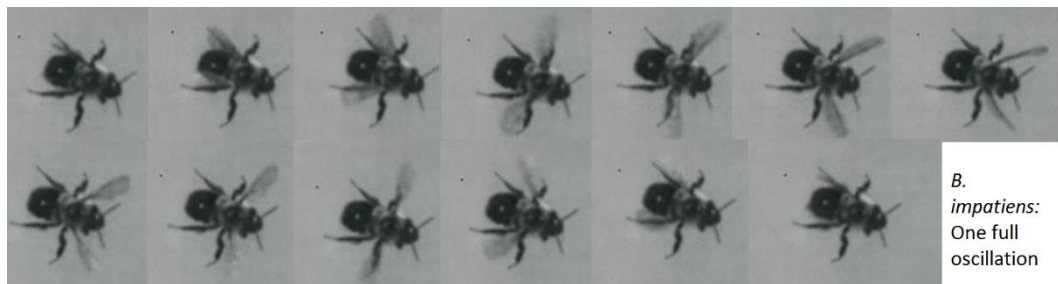


Figure 6: One full oscillation. Frame rate= 2,500 frames per second

We did not see any interesting trends between flight muscle ratio and stroke amplitude, nor did we see interesting trends with flight muscle ratio and wingbeat frequency.

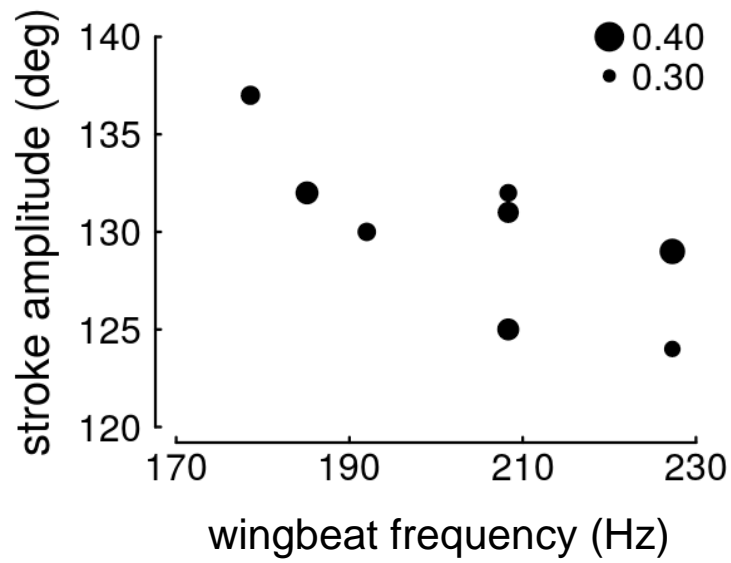


Figure 7: Wingbeat frequency (Hz) vs. stroke amplitude (deg). Size of point indicates flight muscle ratio. Legend located in top right corner.

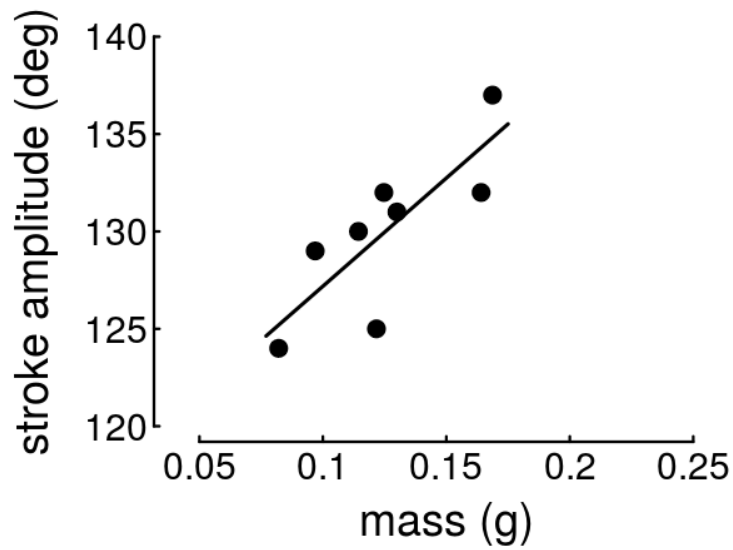


Figure 8: Direct relationship between mass(g) and stroke amplitude (deg)  $p=0.003$   $R^2=0.70$



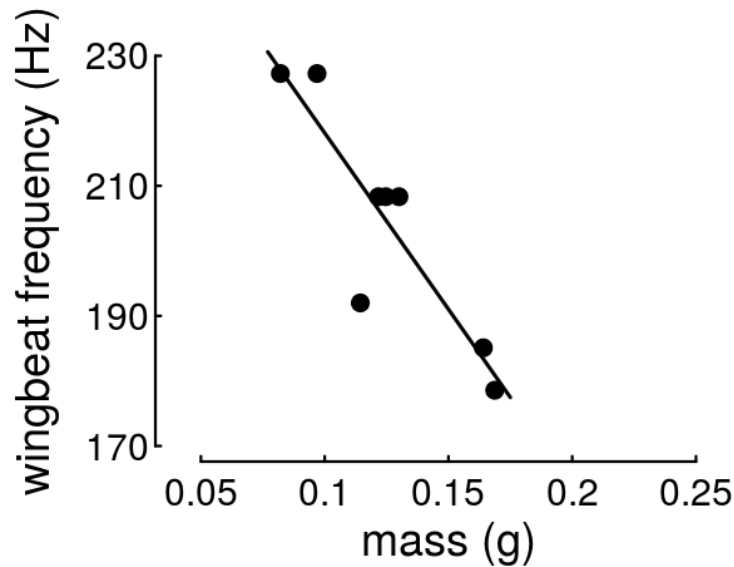


Figure 9: Indirect relationship between mass(g) and wingbeat frequency (Hz).  $p=0.017$   $R^2=0.580$

We had one additional weight-lifting method that we gathered two kinematics data for. This bee lived for four days with a single weight attached to its abdomen. On the fourth day, we recorded kinematics data with the weight attached. After recording the weighted flight, we removed the weight and allowed the bee to live for two days before we performed another flight trial. Wingbeat frequency stayed the same for both trials (~180 Hz), but we saw a noticeable difference in the stroke amplitudes. When the bee was weighted, it had an average stroke amplitude of 140 degrees and when it was unweighted, it had an average stroke amplitude of 130 degrees. If we recall Figure 8, we saw that as mass increased, stroke amplitude increased. While this appears to be a simple answer, it would still be interesting to see if flight muscle is changing because a bee is carrying a weight for most its life.



Figure 10: Bee (weighted). Superimposed images of the up-stroke and down stroke. Average stroke amplitude of 140 degrees.



Figure 11: Bee (unweighted). Superimposed images of the up-stroke and down stroke. Average stroke amplitude of 130 degrees.

## Where do we go from here?

Since we know that bumblebee flight muscle varies with bumblebee age, it would be interesting to know whether this is caused by an endogenous mechanism or if it is the result of regular conditioning. In the short-term, we plan start controlling for age and perform more single-bead weight attachments to see how this affects kinematics and flight muscle ratio. In the long-term, we will perfect the weight-lifting method, and test different species of bees- particularly wild bees. Exercise research has potential to teach us great things about bumblebees. If flight muscle is maintained because of exercise, how do these bees exercise? If an endogenous mechanism maintains it, how is this mechanism regulated? Because this tissue has a key role in bumblebee foraging behaviors, it essentially has a key role in bumblebee survival and could be important in conserving bumblebee populations.

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*Figure 1.* Nerves leading from the second thoracic ganglion of *Bombus* to the longitudinal and dorsoventral flight muscles. Adapted from “Studies on the flight mechanism of insects—II. The innervation and electrical activity of the fibrillar muscles of the bumble bee, *Bombus*,” by K. Ikenda and E.G. Boettiger, 1965, *Journal of Insect Physiology*, 11(6), 781. Copyright 1965 by Pergamon Press Ltd.

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*Figure 4.* Mass(g) vs. flight muscle ratio. M. Dillon and K. Alles (2017).

*Figure 5.* Stroke amplitude. K. Alles (2017).

*Figure 6.* One full oscillation. K. Alles (2017).

*Figure 7.* Wingbeat frequency (Hz) vs. stroke amplitude (deg). M. Dillon and K. Alles (2017).

*Figure 8.* Mass(g) vs. stroke amplitude (deg). M. Dillon and K. Alles (2017).

*Figure 9.* Mass(g) vs. wingbeat frequency (Hz). M. Dillon and K. Alles (2017).

*Figure 10.* Weighted Bee. M. Dillon and K. Alles (2017).

*Figure 11.* Unweighted Bee. M. Dillon and K. Alles (2017).

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