

**Neuromuscular Control of Aerodynamic Power Output via
Changes in Wingbeat Kinematics in the Flight Muscles of
Ruby-throated Hummingbirds (*Archilochus colubris*)**

by

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Abstract

While producing the highest power output of any vertebrate, hovering hummingbirds must also precisely modulate the activity of their primary flight muscles to vary wingbeat kinematics and modulate lift production. By examining how electromyograms (EMGs) and wingbeat kinematics of hummingbirds change in response to varying aerodynamic power requirements during load lifting trials and air density reduction trials, we can better understand how aerodynamic power output is modulated via neuromuscular control. During both treatments increased lift was achieved through increased stroke amplitude, but wingbeat frequency only increased during air density reduction trials. These changes in wingbeat kinematics were matched by increased EMG intensities as aerodynamic power output requirements increased. Despite the relative symmetry of the hovering downstroke and upstroke, the timing of activation and number of spikes per EMG burst were consistently different in the supracoracoideus compared to the pectoralis, likely reflecting differences in muscle morphology.

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Chapter 1

Introduction

1.1 Locomotion

Locomotion is an important aspect of an animal's life. Most animals have to walk, swim, fly, or crawl in order to forage for food, to find mates, and to escape from predators. Therefore, locomotion is very important for an animal's fitness (Altshuler & Dudley, 2002). Locomotion is controlled by the neuromuscular system. Skeletal muscles, activated via neural input, power the movement of limbs (Askew & Marsh, 1997). In addition to powering movement, skeletal muscles are also capable of modulating power output by varying the recruitment of motor units. A motor unit consists of a single motor neuron and all the muscle fibres it innervates (Gage & Moore, 2009). The motor unit is the functional unit in varying mechanical power output. Most animals have different fibre types that can be activated in order to perform activities of varying intensities. For example, fish recruit slow oxidative fibres while swimming at low speeds and recruit fast glycolytic fibres at higher speeds (Jayne & Lauder 1994). Animals that have muscles composed of multiple fibre types can increase power output by these muscles by recruiting more motor units and by recruiting different fibre types. Some muscles are specialized to perform a specific function and they may be composed of only one fibre type, in which case the number of motor units that are activated can be changed in order to vary power output rather than recruiting different fibre types (Ellerby & Askew, 2007; Tobalske et al., 1997).

Hummingbirds have the highest wingbeat frequencies, metabolic rates, and sustained power output requirements of any flying vertebrate (Suarez, 1992). Although hummingbirds have such high metabolic demands they are still exceptionally manoeuvrable with very precise control over power modulation. Hummingbirds must modulate muscle mechanical power output as aerodynamic power output requirements change. For example, the aerodynamic power output requirements for flight are higher if a bird's mass increases. Since hovering flight of hummingbirds represents an extreme for powered flight, understanding the strategies they

employ to increase muscle mechanical power output can provide insight into how power production can be modulated when muscles operate at very high frequencies, a scenario which limits the amount of time available for muscles to do work.

1.2 Wingbeat Kinematics

Hummingbirds must adjust their aerodynamic power output when changing their flight speed, during take-off or landing and while hovering at a feeder. Aerodynamic power output is altered via changes in wingbeat kinematics.

Two of the most important wingbeat kinematic variables responsible for varying aerodynamic power output are wing stroke amplitude and wingbeat frequency. Stroke amplitude is the total angular distance from the bottom of a downstroke to the top of the upstroke during each half stroke (Altshuler & Dudley, 2003; Altshuler et al., 2010). Wingbeat frequency is the number of times the hummingbird beats its wings in one second; one wingbeat is a complete cycle of the wing. The changes in wingbeat kinematics are dependent on multiple factors; such as, mechanical power output of flight muscles, the environment, and wing morphology. An increased aerodynamic power output requirement can be obtained by increasing wingbeat frequency and/or stroke amplitude. Wingbeat kinematics are tightly regulated by muscle mechanics. Muscle fibres operate at the optimal contraction frequency and shortening velocity in order to maximize mechanical power output (Josephson, 1993). Wingbeat kinematics are also dependent on wing morphology. Birds that have a lower wing loading encounter lower aerodynamic power output requirements (Chai & Dudley, 1999). Wing loading is the ratio of the weight of the bird to wing area. Birds with relatively larger wings generally have smaller stroke amplitudes (Altshuler & Dudley, 2010). Smaller birds with small wings tend to have higher wingbeat frequencies and studies have confirmed that wingbeat frequency scales negatively with body mass (Stella et al., 2008; Altshuler & Dudley, 2003). The quality of a bird's wings also

influences wingbeat kinematics. For example, birds that are moulting have a reduced wing area which limits hovering capabilities and lowers the wing's ability to generate lift. Therefore, moulting birds have to increase their wingbeat frequency and or stroke amplitude (Chai & Dudley, 1999). These changes in wingbeat kinematics are attained through changes in neural input, which can be analyzed using electromyography of flight muscles.

1.3 Flight muscles

Among birds, the two main flight muscles are the pectoralis, which powers the downstroke, and the supracoracoideus, which powers the upstroke. In most birds the pectoralis is about five times the size of the supracoracoideus. However, in hummingbirds the supracoracoideus is half the size of the pectoralis (Greenwalt, 1962). The pectoralis originates from the keel of the sternum and dorsally from the ribs and it attaches to the humerus of the wing at the deltopectoral crest. The pectoralis is a bipennate muscle composed of long fibres that have a greater capacity for length change compared to shorter fibres (Wilson and Lichtwark, 2011). The supracoracoideus lies deep to the pectoralis (Biewener, 2012). It originates from the keel of the sternum and inserts via its long tendon dorsally at the shoulder, which passes through the coracoid process of the shoulder (Biewener, 2011). The supracoracoideus is a bipennate muscle with shorter fibres. A muscle that is composed of shorter bipennate fibres is associated with a greater capacity for force production relative to a similarly sized muscle that is composed of longer bipennate muscle fibres (Wilson and Lichtwark, 2011). Force production is more efficient when there is elastic storage of energy (Roberts, 2002). The tendons at either ends of muscles play a significant role in storage of elastic energy. In fact, muscles with long tendons and short fibres are ideal for the storage and return of elastic energy (Roberts, 2002).

Hummingbirds are unique among birds because they are the only group capable of sustained hovering. Hummingbirds are able to hover because the upstroke, which is powered by the supracoracoideus, is capable of generating a substantial amount of lift. Hummingbirds are able to reverse their wing during the upstroke; such that the leading edge is always on top. They also rotate their wing along the long axis with wing turnaround at the end of each half stroke (Tobalske, 2010). The pectoralis and supracoracoideus muscle of hummingbirds contribute to total power output more equally. Therefore, the pectoralis and supracoracoideus of hummingbirds are more functionally similar than in other birds.

The wing muscles of hummingbirds modify the orientation of the wing; therefore, they may influence aerodynamic power output. However, due to the small size of these muscles, they probably contribute very little mechanical power output. In addition, it has been difficult to relate activity of these muscles to specific kinematic changes (Biewener, 2011). Hence, studies generally focus on the two main flight muscles, which produce most of the mechanical power output.

1.4 Varying Mechanical Power Output

Power can be measured at different levels. The first level is metabolic power input. Metabolic power input is the rate at which animals expend chemical energy to fuel their muscles. This metabolic energy is harnessed to generate mechanical power output with certain efficiency, some of the energy being lost as heat. Mechanical power output is a measure of the rate at which muscles do work. The amount of mechanical work a muscle generates is equal to the product of the force and the shortening velocity (Askew & Marsh, 1998). It is the muscles that act on the skeleton and feathers, which results in movements. The mechanical power output is converted into aerodynamic power output at an assumed constant rate of efficiency (Tobalske, 2007).

Therefore, varying aerodynamic power output inevitably changes the mechanical power output requirements. Aerodynamic power output is equal to the sum of the costs associated with lift production, overcoming drag on the wings, overcoming drag on the body, and the kinetic energy required to accelerate the wings. Mathematical models have been developed that permit estimation of aerodynamic power outputs based on morphological parameters and wingbeat kinematics (Ellington, 1984; Rayner, 1979). When aerodynamic power output is calculated using the mathematical models, mechanical power output can be estimated using the assumed rate of efficiency in the transfer of energy between aerodynamic and mechanical power output.

There are many strategies that can be employed in order to vary mechanical power output. Muscle fibre activation influences: i) the amount of time spent shortening or lengthening, ii) the amount of force produced, and iii) the velocity of shortening. In order for a muscle to generate more mechanical power output the timing and intensity of muscle activity can be increased to recruit motor units temporally or spatially. To increase mechanical power output animals can recruit faster contracting muscles or they can recruit a greater number of motor units (Jayne & Lauder 1994). Some animals, such as budgies, keep their muscles inactive in order to reduce mechanical power output during bounding flight (Tobalske et al., 1993). Finally, the strain trajectory of muscles can be changed by altering the amount of time spent shortening or lengthening (Askew & Marsh, 1998). Generating the net power output is dependent on length-force and velocity-force relationships, as well as the kinetics of activation and deactivation.

1.5 Electromyography

Electromyograms (EMGs) are extracellular recordings of the actions potentials from an entire muscle. Therefore, the intensity of EMG recordings is proportional to the number of active

motor units and the amount of mechanical power output generated (Ellerby & Askew, 2007; Tobalske et al., 1997).

Electromyogram recordings (EMGs) of vertebrates are usually compound waveforms due to the activation of different muscle fibre types and asynchronous recruitment of motor units. This complexity makes it difficult to positively relate EMG waveforms to the specific muscle activation characteristics, and thus features of muscle contractile activity, that they reflect. Studies examining muscles and muscle groups which display simple EMG waveforms provide a means to more explicitly relate characteristics of EMGs with variation in mechanical power output. Studying muscles composed of one fibre type would eliminate one of the factors that result in complex EMGs. However, EMG recordings of flight muscles in zebra finches, cockatiels, and budgerigars as they flew at various speeds, were still quite complex despite the uniform fibre type makeup of their flight muscles (Ellerby & Askew, 2007). Interestingly, electromyogram recordings of hummingbirds are unusual among vertebrates because they consist of a small number of discrete spikes, which are readily interpretable (Altshuler et al., 2010; Tobalske et al., 2010). The simple waveforms observed in hummingbirds are the summed result of two different properties of hummingbird flight muscles. Firstly, hummingbirds only have one type of muscle fiber, fast-twitch oxidative glycolytic fibers (Welch & Altshuler, 2009). Secondly, the flight muscles contract at the highest frequencies of any flying vertebrate. Therefore, the window for activation is the smallest in comparison to any other vertebrate flight muscles. The limited time available for the activation of muscles leads to the muscle fiber action potentials being more temporally synchronized across motor units; a feature that is unique to hummingbirds (Altshuler et al., 2010).

In cockatiels and budgerigars it has been found that EMG activity begins in the pectoralis prior to the downstroke and continues about halfway into the downstroke until the maximum

peak force is generated (Hedrick et al., 2003). In hummingbirds the activation of the pectoralis muscle begins before the downstroke, but deactivation also ends before the start of the downstroke. Similarly, the deactivation of the supracoracoideus ends prior to the upstroke (Altshuler et al., 2010; Tobalske et al., 2010). All muscles require time to relax; with such high wingbeat frequencies, if the EMG activity continued into the downstroke or upstroke it negatively impact the amount of net positive work done by the muscle (Tobalske et al., 2010). Specifically, if a muscle does not completely relax then some tension will remain, resulting in greater negative work during the lengthening phase (Josephson, 1993; Tobalske et al., 2010).

Since hummingbirds have relatively simple EMG recordings, which can be simultaneously recorded along with wingbeat kinematics while they are flying, they are excellent model organism to study how power output is modulated through neuromuscular control and how that translates to changes in wingbeat kinematics.

1.6 Altering Aerodynamic Power Output Requirements

As mentioned above, EMGs record electrical potential differences of the extracellular milieu resulting from the transmission of action potentials along the membranes of groups of muscle fibers. Therefore, one cannot decipher the exact number of motor units that are active from an EMG trace. By increasing aerodynamic power output conclusions can be drawn about how EMGs change with increasing power demands. Since relative intensities reflect the relative number of active motor units in a given area of muscle tissue, which is positively correlated with force production, conclusions can be made about how activation of motor units varies across changing power demands. Therefore, in order to obtain meaningful data from EMGs it is necessary to alter mechanical power output requirements via increases in aerodynamic power output requirements.

Air density reduction trials using heliox are often employed in order to increase aerodynamic power output requirements. Heliox is composed of 21% oxygen and 79% helium. Therefore, the partial pressure of oxygen is maintained, ensuring metabolic power production is not limited. Yet, heliox is less dense than ambient air (Chai and Dudley, 1997). Less dense air reduces the amount of drag the birds have to overcome, but it also reduces the amount of lift generated by the wings. Reduced air densities require compensatory changes in wingbeat kinematics to achieve sufficient lift thus requiring higher mechanical power output (Altshuler and Dudley, 2003; Chai and Dudley, 1997). Altshuler et al. (2010) obtained EMGs of the pectoralis muscle and wingbeat kinematics of Anna's hummingbirds while air density was decreased using progressive heliox replacement of ambient air. The birds increased their aerodynamic power output by progressively increasing stroke amplitude with moderate increases in wingbeat frequency as air density decreased. EMG amplitude increased with decreasing air density, while the number spikes per burst did not change. Other studies that employed heliox trials also found large increases in stroke amplitude and moderate increases in wingbeat frequency as air density decreased in rufous and ruby-throated hummingbirds. (Altshuler & Dudley, 2003; Chai & Dudley, 1995). If there is an increase in stroke amplitude and wingbeat frequency, then the wing must be accelerated more rapidly through each half stroke. Air density is directly proportional to the drag force on the wings (Yin & Luo, 2010). Heliox trials reduce air density, which also decreases the drag on the wings. Whether the increased acceleration is due to actively modulated increases in muscle force production, or the result of reduction in passive drag on the wing is unclear.

Another method that can be employed to increase aerodynamic power output is to have birds lift weights. Attaching loads increases wing loading and thus challenges birds to increase power output. Because such trials can be done in ambient air the results can be analyzed without

having air density as an additional potentially confounding variable. Furthermore, examination of the effects of added weight on flight kinematics and muscle activity is ecologically relevant because ruby-throated hummingbirds can gain a substantial amount of weight during individual feeding events and over a few days as part of pre-migratory fattening. In Ontario, mass measurements of free ranging RFID chip tagged ruby-throated hummingbirds were obtained during feeding events. Mass increases of more than 0.5 g ($\approx 15\%$ of body mass) were routinely observed over the course of single feeder visitation events (K. C. Welch, personal communication). Calder et al. (1990) reported a total intake of sucrose solution that was 34% of an individual's mass in broad-tailed hummingbirds (*Selasphorus platycercus*) twenty minutes before flying to roost. During their pre-migratory period ruby-throated hummingbirds can nearly double their mass (Norris et al., 1957). The increase in mass is largely due to deposits of fat (Norris et al., 1957). Rufous hummingbirds increased their mass on average from 3.2 to 4.6 grams on refuelling stopovers on their migratory route. The first 0.3 grams that was gained was not fat and it was hypothesized that it was most likely lean tissue deposited in order to rebuild muscle that may have been catabolised during migration (Carpenter et al., 1993). However, the remaining increase in mass was entirely lipid (Carpenter et al., 1993). Therefore, the mass gained during the migratory period is mainly fat, rather than muscle mass. An increase in body mass increases the weight of the birds and they have to generate more lift to fly, which increases their power output requirement. Similarly, as birds feed they gain mass while hovering at a feeder and they have to fine tune their wingbeat kinematics to match the changing aerodynamic power output requirement via changes in their neuromuscular control.

Aerodynamic power output requirements vary as a function of flight velocity. Most recent studies on rufous hummingbirds, budgerigars and zebra finches have found a U shaped relationship between mechanical power output and forward flight speed (Tobalske et al., 2010;

Askew & Ellerby, 2007). A similar U shaped pattern was also observed for the relationship between normalized EMG amplitude and flight speed in rufous hummingbirds (Tobalske et al., 2010). Therefore, flying at very low speeds and very high speeds is more expensive than flying at intermediate speeds. Recent evidence suggests that power output requirements for backward flight may vary similarly as a function of flight velocity. Sapir & Dudley (2012) reported that rates of oxygen consumption are higher during both forward flight and backward flight up to a speed of 4.5m/s than during hovering flight. Hummingbirds also perform tasks that require them to briefly generate maximal power output; for example, during an escape response. Asymptotic maximal load lifting assays are a common method employed to explore how wingbeat kinematics and neural input change when the bird is challenged to produce the maximum aerodynamic power. Altshuler et al. (2010) found that Anna's hummingbirds increase EMG amplitude and the number of spike per burst, which translates to an increase in both stroke amplitude and wingbeat frequency in order to generate maximal power output. They interpreted the increase in EMG amplitude as spatial recruitment of motor units and the increase in spike numbers as temporal recruitment of motor units. Maximal load lifting trials differ from the sub-maximal trials because they are not sustainable. Looking at the kinematics of maximal load lifting allows us to look at the upper constraints on aerodynamic power output.

1.7 Calculating Mechanical Power Output

The amount of mechanical work a muscle generates is equal to the product of the force and the shortening velocity (Askew & Marsh, 1998). *In vitro*, mechanical power output is often measured using the work loop technique (Josephson, 1993; Askew & Marsh, 1997; Hedrick et al., 2003). *In vivo*, mechanical power output is often calculated using aerodynamic power requirements (Chai & Dudley, 1995; Chai & Dudley, 1997; Altshuler et al., 2010) or by implanting sonomicrometry muscle length transducers along with strain gauges to measure force

(typically implanted on the deltopectoral crest of the humerus, the site of insertion of the pectoralis; Hedrick et al., 2003; Tobalske et al., 2010). In the method using aerodynamic power requirements, the mechanical power output required by the muscles is calculated assuming that it needs to match the of aerodynamic power output requirements. There are different mathematical models that determine aerodynamic power output using wingbeat kinematics and wing morphology (Ellington, 1984; Rayner, 1979). These models have been used in research that looks at how wingbeat kinematics change as a result of differing power requirements and to estimate the mechanical power output (Chai & Dudley, 1995; Hedrick et al., 2003). Only a limited number of experiments have looked at how mechanical power output is modulated via wingbeat kinematics through changes in neuromuscular control during hovering flight (Hagiwara et al., 1968; Altshuler et al., 2010; Tobalske et al., 2010).

1.8 Goals of the Study

The locomotion of most animals typically involves cyclical oscillations of limbs , whether it is swimming, climbing or flying. The use of electromyography along with high speed videography helps shed light on how neural input controls locomotion.

This is the first study that has combined high speed videography of sub-maximal, maximal load lifting, and air density reduction trials with simultaneous recording of EMGs from the pectoralis and supracoracoideus in ruby-throated hummingbirds (*Archilochus colubris*). Through the use of load lifting trials and air density reduction trials this study investigates how changes in EMG activation patterns relate to variation in wingbeat kinematics in order to increase aerodynamic power output requirements in ruby-throated hummingbirds. Air density reduction trials and heliox trials examine sustained hovering; whereas, maximal load lifting assays assess transient maximal power output capabilities. EMGs have not been previously collected on ruby-throated hummingbirds; therefore, this study will help elucidate whether there

are interspecific differences in how hummingbirds modulate wingbeat kinematics when challenged to increase aerodynamic power output. Heliox studies that used Anna's (Altshuler et al., 2010) and ruby-throated hummingbirds (Chai & Dudley, 1995; Chai & Dudley, 1997) reported that stroke amplitude increased substantially with moderate increases in wingbeat frequency as a function of decreasing air density. In Anna's hummingbirds the increase in wingbeat kinematics was matched by an increase in normalized EMG amplitude as air density decreased, but spike number did not change (Altshuler et al., 2010). During maximal load lifting trials both EMG area and number of spikes increased (Altshuler et al., 2010). A separate study examining a different hummingbird species, rufous hummingbirds (*Selasphorus rufus*), found that lifting progressively greater sub-maximal loads was associated only with increased stroke amplitude but no change in wingbeat frequency (Wells, 1993).

Across air density reduction trials, I hypothesize that ruby-throated hummingbirds produce greater aerodynamic power output predominately via increases in stroke amplitude, with moderate increases in wingbeat frequency. I expect that the increase in wingbeat kinematics is matched by an increase in normalized EMG area and normalized spike amplitude during sustained hovering. Prior studies suggest that the high frequency of hummingbird wingbeats constrains the window of motor unit activation, thus resulting in the observed small number of spikes per burst characteristic of Anna's and rufous hummingbird pectoral EMGs. The wingbeat frequency of ruby-throated hummingbirds is even higher than that of either rufous or Anna's hummingbirds; therefore, I predict that the number of spikes per burst recorded from ruby-throated hummingbirds will be even more temporally constrained and thus will consist of only one spike per burst. (Chai & Dudley, 1995; Chai & Dudley, 1997). Simultaneous EMG recordings of both the pectoralis and the supracoracoideus muscles provide insight into whether activation patterns are similar between the muscles. I hypothesize that the activation patterns of

the pectoralis and supracoracoideus muscles would change in a similar fashion in relation to EMG characteristics across all the different treatment levels, consistent with their need for more similar power output as a result of hummingbirds more symmetrical half strokes.

Load lifting trials consist of both sub-maximal load lifting trials and maximal load lifting trials. I predict that only stroke amplitude increases across sub-maximal load lifting trials. I predict that both stroke amplitude and wingbeat frequency increase during maximal load lifting trials. I expect that the increase in wingbeat kinematics is matched by an increase in normalized EMG area and normalized spike amplitude across sub-maximal loads. During maximal load lifting trials I expect normalized EMG area, normalized spike amplitude and number of spikes per burst increases.

As noted earlier, heliox studies involving Anna's and ruby-throated hummingbirds reported that both stroke amplitude and wingbeat frequency increased significantly. However, rufous hummingbirds that lifted progressively greater sub-maximal loads only increased stroke amplitude. These distinct results suggest either that there are interspecific differences in the way hummingbirds alter wingbeat kinematics to vary aerodynamic power output or that changes in wingbeat kinematics differ as a result of the nature of the lift generating challenge. Through the use of both hypodense trials along with load lifting trials on the same individual this study investigates whether the same bird increases power output similarly in both experiment types in relation to wingbeat kinematics and EMG characteristics. More specifically, I will test whether lower air density makes it easier for birds to increase wingbeat kinematic variables without an increase in the intensity of neuromuscular activation. I hypothesize that patterns of variation in wingbeat kinematics differ between the two experiment types because air density reduction trials reduce the drag on the wings, which may make it easier to accelerate the wing further for a given amount of force. Therefore, I expect that both stroke amplitude and wingbeat frequency increase

in air density reduction trials, but I expect only stroke amplitude to increase in sub-maximal load lifting trials.

Chapter 2
Materials and Methods

2.1 Experimental Animals

Four adult ruby-throated male hummingbirds (*Archilochus colubris*) were captured in Scarborough, ON, Canada. The birds were housed individually in 61 by 61 by 61 cm cages. They were fed nectar plus *ad libitum*. The birds were numbered 44, 45, 46, and 47 and the birds' masses were 2.73g, 2.82g, 2.94g, and 2.75g respectively. The mass reported for each bird is an average of the bird's mass measured at the beginning and at the end of the experiment.

2.2 Experimental Design

The experiment was conducted in a testing arena that was 61cm in width by 62.2cm in length by 76.2cm in height. The birds were trained to perch, fly, feed, and lift weights. They fed from a 1ml syringe and once the birds were comfortable feeding, access to the feeder was restricted by occluding it with a shield. The birds were trained to feed only when permitted by removing the shield at approximately 10-20 minute intervals. Muscle activation and wingbeat kinematics were studied for all four birds under the following conditions: (1) hovering at a feeder (in ambient air without any load attached), (2) hovering at a feeder in progressively less dense normoxic (heliox-ambient) air mixtures, (3) hovering at a feeder while lifting progressively heavier sub-maximal loads, and (4) hovering briefly while lifting maximal loads (Figure 1).

2.3 Surgery

Muscle activation was recorded for both the pectoralis and supracoracoideus using electromyography. The materials and method employed for the collection of EMGs was the same as those used in Altshuler et al. (2010). Prior to the implantation of the electrodes birds were anaesthetized using vaporized isoflurane. The birds were placed on a heating pad and a mask was placed over their bills. The oxygen flow rate was set to 0.3 L/minute. The concentration of the isoflurane was set to 1.5% and it was increased progressively until the bird stopped moving, and the pedal withdrawal reflex was absent. The concentration of isoflurane never had to be

increased above 2.5%. The implantation of the electrodes was done while the birds were under anaesthesia. Two bipolar electrodes were made from a pair of 0.08 mm diameter bifilar HML-insulated silver wires (California Fine Wire Company, Grover Beach, California). The tips were offset by approximately 0.5mm and stripped of insulation for the first 0.5mm of each lead. The end of each bifilar electrode was inserted into a 26 gauge needle and bent 180 degrees in order to form a hook. A third, monofilar, insulated silver wire was stripped of insulation for the first 0.5mm of the lead to form a ground electrode. The tip of the ground electrode was also inserted into a needle and bent back in order to form a hook. One of the bipolar electrodes was inserted into the left pectoralis muscle (Figure 2A). The wire was held in place using tweezers, while the needle was removed. The hook at the end of the wire kept the wire embedded in the muscle. The wire was sutured (6-0, silk suture) to the skin above the pectoralis. The same procedure was followed for the implantation of the electrode into the left supracoracoideus muscle; however, the needle was inserted more deeply. The wire from the supracoracoideus was also sutured to the skin above the pectoralis muscle (Figure 2B). The ground electrode was implanted on the back under the skin. The two bipolar electrode wires were fed cranially and dorsally over the left shoulder joint and then caudally along the back, running near the point of insertion of the ground electrode. All three wires were then sutured together on the intervertebral fascia on the dorsal side of the animal. Figure 2 illustrates the approximate location of placement of each of the two bipolar recording electrodes. Once the surgery was complete the anaesthesia was turned off and the birds were given a few minutes to wake-up and recover.

2.4 Data Collection

- 1) After the surgery was complete the bird was placed in the testing arena. Two recordings were collected while the bird fed at the artificial flower in ambient air without a load

attached. These initial recordings served as baseline measurements for kinematic variables and as controls for the EMG traces.

2) The testing arena contained a Galton whistle that was blown in order to determine air density. The fundamental frequency of a whistle is dependent on the density of the air. The testing arena was continuously filled with heliox and the whistle was blown immediately after the bird fed. When the whistle was blown it was recorded using Raven Lite (v.1.0, Cornell Bioacoustics Research Program Software). The sound recordings were then analyzed using a Matlab (v.7.12.0, Mathworks Inc.) script, provided by Douglas Altshuler, which determined the frequency of the whistle. The changes in fundamental whistle frequency were used to determine the air density. The aim of the heliox trials was to reduce the air density from ambient air density of 1.2 kg/m^3 to air densities of approximately 1, 0.9, 0.8 and 0.7 kg/m^3 . The air density reduction trials were accomplished by progressively pumping heliox (21% oxygen and 79% helium) into the arena at a rate of 8.5 L/min. Through trial and error the amount of time required to fill the chamber with heliox was determined and recording were collected while birds hover fed in an air-heliox gas mixture with a density of approximately 1, 0.9, 0.8 and 0.7 kg/m^3 . Once the air density trials were complete the chamber was opened and ambient air was allowed to flow into the testing arena. Two more trials were conducted in ambient air as internal controls that could be compared to the first EMG recordings. These ambient hovering trials, conducted after each treatment, served as internal controls that were important in ensuring that the EMG electrodes had not shifted.

3) Sub-maximal load lifting trials were conducted after the air density trials. Harnesses with a mass of 0.25, 0.5 or 0.75 grams were placed around the necks of birds. Recordings were collected of birds hover feeding while lifting each sub-maximal load. The order of the sub-maximal loads was randomized. It was not considered sustained hovering if the bird was not able

to fly from the perch, feed for a minimum of 2 seconds, and fly back to the perch. If the bird descended to the floor after the feeding I interpreted this to indicate that the bird was only able to lift the load transiently and thus such trials did not qualify as sustained hovering. After the sub-maximal trials were complete another recording was made of the bird feeding without any load attached.

4) Maximal load lifting trials were conducted by attaching a harness attached to a long chain of beads around the neck of the birds. The birds were placed on the bottom of the arena and they flew straight up, which is their natural escape response. As the birds ascended they transiently hovered while lifting a maximal load before descending. Three maximal load lifting trials were recorded. The number of beads that the bird lifted was counted to calculate the load that the bird was able to lift. The trial that resulted in the bird lifting the maximum number of beads was analyzed. Finally, the harness was removed from the bird and another trial was conducted where the bird hover fed at the feeder without a load attached to it.

2.5 Data Analysis

2.5.1. EMG Data

EMG signals were amplified 1000 times using an extracellular amplifier (A-M Systems, Differential AC Amplifier, model 1700), which was set to filter low and high frequency cut-offs of 0.1 Hz and 10 KHz. The analog signals were obtained using an analog-to-digital converter (Digidata 1440A, Molecular Devices, California, United States) sampling at 10000Hz. The EMGs were recorded using Axoscope (v.10.3) The EMG signals were then post-processed. A zero-phase, forth-order high-pass Butterworth filter with a cut-off frequency set at approximately 12 times the wingbeat frequency was used to remove movement artifacts and the mean of the inactive portions of the signal were set to zero. Fifteen consecutive wingbeats were analyzed at each level while the bird was hover feeding in air density trials and sub-maximal load lifting

trials. For the maximal load lifting assays, fifteen wingbeats were analyzed while the bird was lifting the maximum number of beads. EMG area (the rectified area of each EMG burst), EMG amplitude (height of each spike within each burst), EMG onset (start of EMG activity prior to the beginning of the downstroke for the pectoralis and the start of EMG activity prior to the beginning of the upstroke for the supracoracoideus) and number of spikes per burst were calculated using the same Matlab script employed in Altshuler et al., 2010. EMG amplitudes were normalized against the maximum EMG amplitude for each bird across all of its trials. The spike threshold was set to 0.25 times the highest spike amplitude during each run, in order to automate the detection of discrete spikes and determine the number of spikes per burst.

Video recording was stopped by use of disruption of a continuous 5V analog voltage. This analog voltage was also recorded along with the EMG data, providing a means of temporally synchronizing video and EMG data. An additional analog to digital converter was also used, which interfaced with the MIDAS DA program (Xcitex, Massachusetts, United States) to display both the EMGs and videos simultaneously. The trigger was also recorded simultaneously in the MIDAS program environment. This National Instruments (Austin, Texas, US) analog-to-digital converter was not as precise because it only recorded at a 1000 Hz, but it provided an independent means of confirming synchronization of the EMGs and video data.

2.5.1. Kinematic Data

Flight trials were filmed using three high speed video cameras (S-PRI, AOS technologies AG, Baden Daettwil, Switzerland) which recorded at 1000 frames per second at shutter speed of 250 μ s. One camera was placed above the arena (top view), the second camera was placed facing the left side of the bird, and the third camera was placed facing the back of the bird. The videos were recorded to PC using AOS Imaging Studio (version 3.4.7, AOS technologies AG, Baden Daettwil, Switzerland). The wingbeat frequency and stroke amplitude were obtained by

analyzing the videos. Fifteen consecutive wingbeats were analyzed at each level while the bird was hover feeding in air density trials and sub-maximal load lifting trials. For the maximal load lifting assays, fifteen wingbeats were analyzed while the bird was lifting the maximum number of beads. The same wingbeats were analyzed for both wingbeat kinematics and EMG characteristics. The stroke amplitude was determined by measuring the angle between the bottom of the downstroke and the top of the upstroke of the left wing. The wingbeat frequency was calculated by first counting the number of frames it takes to complete one wingbeat. The number of frames was then divided by 1000 to obtain the amount of time it takes to complete one full wingbeat (period). Wingbeat frequency was calculated by using the equation, wingbeat frequency = 1/period.

2.5.3 Mechanical Power Output

The mass of each bird was measured at the beginning and end of each experiment using a digital balance that has a precision of 0.1 mg (MS-104S, Mettler Toledo, Switzerland). The mean of the two measurements constituted the mass of the bird during trials for use in later calculations of power output. The average mass change over the duration of all trials was approximately 0.04 g. Following all trials, wing morphology was determined by taking photographs were taken of each bird's wings while the wings were outstretched against grid paper. The wing images were analyzed using a Matlab script (provided by Douglas Altshuler), which calculated parameters such as wing length, aspect ratio and wing area. The bird's mass, morphology of the wing, kinematic measurements and environmental variables (air density) were incorporated into an aerodynamic model to estimate mechanical power output based on aerodynamic power output requirements (Ellington, 1984).

2.6 Statistics

Data were analyzed using the statistical program, SPSS (v.17.0, IBM, United States). Data were analyzed using repeated measures ANOVA to test for trends in EMG and kinematic parameters as a function of treatment level and to compare supracoracoideus and pectoralis activation patterns. The predictor variables were the levels of air density treatments (1.2, 1.0, 0.9, 0.8, 0.7 kg/m³) or load lifting trials (0.25, 0.50, 0.75 g) with bird ID (bird 44, 45, 46, 47) treated as a random effect. If the data violated the test of sphericity, the Greenhouse-Geisser, Huynh-Feldt, and Lower Bound correction factors were applied to adjust the degree of freedoms and significance values. Lower bound corrected values are reported because the lower bound correction is the most conservative of the three. One-way ANOVA with post hoc analyses were conducted to test for variations among birds. The statistical program R (v. 2.15.1) was used in order to compare whether experiment type, load lifting versus air density reduction, was a significant factor influencing aerodynamic power output. The effects of estimated power output, trial type, and an interaction factor on (normalized EMG area of pectoralis, normalized EMG area of supracoracoideus, stroke amplitude, and wingbeat frequency) was estimated by fitting of a mixed effects, repeated measures design model with ID as a random factor (using the "lme4" package). The significance of model parameters was assessed by comparison against Markov Chain Monte Carlo derived parameter populations (using the "languageR" package). Results were considered significant if the P-values were less than 0.05. All data is presented as mean \pm standard deviation.

Chapter 3

Results

3.1 Regulation of Wingbeat Kinematics via Neural Input Across Varying Aerodynamic Power Output Requirements

The mean wingbeat kinematic and EMG measurements for both flight muscles under load lifting and air density reduction trials are presented in Figure 3. Wingbeat frequencies were lower and stroke amplitudes were higher for bird 44 than the other birds across load lifting trials (see Figure 4) and air density reduction trials (see Figure 5). Bird 46 had higher wingbeat frequencies and lower stroke amplitudes relative to the other birds under both under density reduction trials and sub-maximal load lifting trials. Stroke amplitude increased progressively as the birds lifted heavier loads (0 to 0.75g) from 140.84 ± 11.29 to 157.28 ± 10.75 Hz (see Figure 3A) ($F_{1,4} = 34.46$, $P = 0.01$). During maximal load lifting trials stroke amplitudes often reached the birds' geometrical constraint of approximately 180 degrees (174.26 ± 5.07). Wingbeat frequency did not differ significantly between sub-maximal load lifting trials (see Figure 3B) (54.47 ± 23.32 to 55.99 ± 3.44 Hz when lifting 0 and 0.75 g, respectively; $F_{3,9} = 1.31$, $P = 0.33$). Wingbeat frequency was not significantly greater during maximal load lifting (55.99 ± 2.04 Hz; $F_{1,3} = 1.27$, $P = 0.34$). Among air density reduction trials, stroke amplitude (see Figure 3C) ($F_{1,3} = 17.28$, $P = 0.03$) and wingbeat frequency (see Figure 3D) ($F_{4,12} = 3.47$, $P = 0.04$) increased significantly; however, stroke amplitude never reached values as high as those employed during maximal load lifting assays. When the lowest air density was removed from analysis, in order to test whether it was driving the trends, the wingbeat frequency no longer varied significantly with air density ($F_{3,9} = 1.55$, $P = 0.268$).

The sample traces of the pectoralis and supracoracoideus shown in Figure 6 are direct outputs from the amplifier with analog filter cut-offs of 1Hz and 10 kHz. Normalized EMG area was assumed to reflect the number of motor units that were activated. Normalized EMG area of the pectoralis increased significantly (0.30 ± 0.45 to 0.40 ± 0.10 when lifting 0 to 0.75 g,

respectively) (see Figure 7A) ($F_{1,3} = 46.17$, $P = 0.007$) as birds lifted heavier loads. Normalized EMG area also increased for the supracoracoideus as more mass was lifted (0.31 ± 0.11 to 0.38 ± 0.08 when lifting 0 to 0.75 g, respectively) (see Figure 7A) ($F_{1,3} = 75.50$, $P = 0.003$). EMG area also increased significantly for the pectoralis (0.30 ± 0.45 to 0.58 ± 0.17 across air densities of 1.2 to 0.7 kg/m³, respectively) (see Figure 8A) ($F_{4,12} = 5.92$, $P = 0.007$) and the supracoracoideus muscles (0.31 ± 0.11 to 0.51 ± 0.10 across air densities of 1.2 to 0.7 kg/m³, respectively) (see Figure 8A) ($F_{4,12} = 5.81$, $P = 0.008$) under air density reduction trials. Some studies have used normalized EMG amplitude instead of normalized EMG area as a measure of the number of active motor units, but analysis of normalized EMG amplitude of the largest peak within a burst did not show any trends in relation to lifting heavier loads or progressively reduced air densities ($p > 0.05$).

EMG traces from the pectoralis and the supracoracoideus muscles of ruby-throated hummingbirds are composed of a discrete number of spikes per burst during hovering flight under both load lifting trials and air density reduction trials. The number of spikes did not change significantly during sustained hovering while lifting sub-maximal loads or during air density reduction trials. During sustained hovering, birds exhibited 1-2 spikes per burst. However, this increased to 2.5-3 spikes per burst during maximal load lifting assays. Across all sub-maximal load lifting trials the pectoralis exhibited an average of 1.32 ± 0.28 spikes per burst ($F_{1,3} = 1.27$, $P = 0.34$) and the supracoracoideus exhibited 1.78 ± 0.32 spikes per burst (see Figure 7B) ($F_{1,3} = 2.43$, $P = 0.22$). During maximal load lifting the pectoralis exhibited on average exhibited 2.57 ± 0.38 spikes per burst ($F_{4,12} = 22.39$, $P < 0.001$) and the supracoracoideus on average exhibited 2.8 ± 0.59 spikes per burst ($F_{4,12} = 5.78$, $P = 0.008$). Across all the air density reduction trials the pectoralis exhibited an average of 1.35 ± 0.23 spikes per burst ($F_{4,12} = 2.82$, $P = 0.07$); whereas,

the supracoracoideus exhibited 1.90 ± 0.36 spikes per burst (see Figure 10B) ($F_{4,12} = 27.874$, $P = 0.67$) (see Figure 8B).

The activation of antagonistic muscles were completely out of phase with one another. The activation patterns were also cyclic in nature with very little variation in timing. The pectoralis was activated and deactivated prior to the start of the downstroke. Similarly, the activation of the supracoracoideus started and ended before the upstroke. The timing of activation of the pectoralis muscle prior to the downstroke did not vary significantly as the birds increased aerodynamic power output within load lifting trials, occurring 4.30 ± 0.48 ms prior to the start of the downstroke (the pectoralis was activated $52.64 \pm 5\%$ into the upstroke, where the full upstroke represents 100%) (see Figure 7C) ($F_{1,3} = 1.68$, $P = 0.29$). Similarly the onset of EMGs of the supracoracoideus prior to the upstroke did not vary under load lifting trials, occurring 5.07 ± 0.51 ms prior to the start of the upstroke (the supracoracoideus was activated $44.17 \pm 5.6\%$ into the downstroke, where the full downstroke represents 100%) (see Figure 7C) ($F_{1,3} = 0.697$, $P = 0.465$) or air density trials. Across air density reduction trials the birds did not significantly vary the timing of activation of the of the pectoralis muscle (see Figure 8C) ($F_{4,12} = 1.21$, $P = 0.36$) or the supracoracoideus muscle (see Figure 8C) ($F_{1,3} = 1.10$, $P = 0.371$). Therefore, the timing of the activation of the flight muscles was not dependent on power output requirements.

3.2 Comparing the Activation Patterns of the Supracoracoideus and the Pectoralis

EMG measurements were compared between the pectoralis and the supracoracoideus muscles. The mean values of the normalized EMG area, number of spikes per burst and timing of EMG onset are presented for both muscles under air density reduction trials in Figure 7 and load lifting assays in Figure 8. The difference between the mean values of all three EMG parameters of the pectoralis and supracoracoideus are shown in Figures 9 and 10.

On average, normalized EMG area did not significantly differ between the two muscles within load lifting trials (see Figure 9A) ($F_{1,6} = 0.025$, $P = 0.88$) or air density reduction trials (see Figure 10A) ($F_{1,6} = 0.770$, $P = 0.414$). Hence, the activation of motor units, relative to the maximal activation observed at any point during the trials, did not differ between the two primary flight muscles.

The number of spikes per burst was significantly greater in the supracoracoideus than the pectoralis within both air density trials and load lifting trials. Across all sub-maximal load lifting trials the supracoracoideus exhibited 0.46 more spikes per burst (1.3 times as many spikes per burst) than the pectoralis. (see Figure 9B) ($F_{1,6} = 14.36$, $P = 0.009$). During maximal load lifting the supracoracoideus exhibited on average 0.23 more spikes per burst (1.09 times as many spikes per burst) as the pectoralis ($F_{1,6} = 14.36$, $P = 0.009$). Across all the air density reduction trials the supracoracoideus on average had 0.55 more spikes per burst (1.4 times as many spikes per burst) as the pectoralis (see Figure 10B) ($F_{1,6} = 27.874$, $P = 0.002$).

The timing of EMG onset was significantly earlier in the supracoracoideus than in the pectoralis, measured with respect to the relevant wingbeat transition. During load lifting trials, the supracoracoideus was activated, on average 0.77 ± 0.03 ms earlier, relative to the wingbeat transition than the pectoralis (8.47 ± 0.6 % earlier relative to the wingbeat transition) (see Figure 9C) ($F_{1,6} = 8.74$, $P = 0.025$). During the air density reduction trials, the supracoracoideus was activated 1.05 ± 0.21 ms earlier, relative to the wingbeat transition than the pectoralis (11.53 ± 2.29 % earlier relative to the wingbeat transition) (see Figure 10C) ($F_{1,6} = 6.42$, $P = 0.04$).

3.3 Comparing Air Density Trials and Load Lifting Trials

In order to facilitate comparison between trial types mechanical power output produced by each bird during each trial was calculated using equations published by Ellington (1984). Kinematic and EMG data were subsequently evaluated as a function of power output, rather than

mass lifted or air density. When plotted as a function of mechanical power output the normalized EMG area was not significantly different between sub-maximal load lifting trials and air density reduction trials for the pectoralis (see Figure 11) ($P = 0.13$) or the supracoracoideus ($P = 0.13$) (see Figure 12). Similarly, in order to generate the same mechanical power output the stroke amplitude (see Figure 13) ($P = 0.77$) and wingbeat frequency (see Figure 14) ($P = 0.88$) employed by the hummingbirds did not differ between the two experimental types. The results suggest that in order to attain a given aerodynamic power output hummingbirds use similar wingbeat kinematics. When birds generate the same normalized EMG area of the pectoralis (see Figures 15 & 16) or the supracoracoideus (see Figures 17 & 18) the stroke amplitude and the wingbeat frequency were not significantly different between sub-maximal load lifting trials and air density reduction trials ($P > 0.1$). Therefore, results suggest that for a given amount of activated motor units the wingbeat frequencies and stroke amplitudes generated are similar between air density reduction trials and sub-maximal load lifting trials.

Chapter 4

Discussion

4.1 Wingbeat Kinematics

In order to hover, birds must overcome drag and generate sufficient lift. Additional loads and reduced air densities increase the amount of mechanical power output birds have to generate in order to sustain hovering. A given power output can be attained by various combinations of wingbeat frequency and stroke amplitude; however, stroke amplitude is geometrically limited at approximately 180 degrees and wingbeat frequency is physiologically limited (Chai et al., 1997). The results from this study suggest that birds with relatively lower stroke amplitudes compensate with higher wingbeat frequencies and *vice versa*.

Patterns of variation in stroke amplitude in relation to air density were consistent with previous findings in ruby-throated hummingbirds (Altshuler & Dudley, 2003; Chai & Dudley, 1995; Chai & Dudley, 1997). Under both load lifting trials and air density reductions trials stroke amplitude increased significantly. Significant increases in stroke amplitude with increasing aerodynamic power output requirements have been observed in Anna's (*Calypte anna*) (Altshuler et al., 2010), rufous (*Selasphorus rufus*) (Tobalkse et al., 2010), and ruby-throated hummingbirds (*Archilochus colubris*) (Chai & Dudley, 1995; Chai & Dudley, 1997; Altshuler & Dudley, 2003). Hummingbirds possess the ability to increase stroke amplitude during sustained hovering and forward flight, in order to match aerodynamic power output requirements, while maintaining the same wingbeat frequency. Across species, hummingbirds that live at higher altitudes also have higher stroke amplitudes in order to compensate for their hypodense, hypoxic environments (Altshuler et al., 2004). Higher elevations also require the bird to generate greater mechanical power output in order to sustain hovering. In this study, during maximal load lifting birds reached their geometrical limit of 180 degrees, which usually occurs when the birds try to produce their maximum aerodynamic power output (Altshuler & Dudley, 2003; Chai & Dudley, 1995; Chai & Dudley, 1997).

The wingbeat frequencies observed in the ruby-throated hummingbirds across all sustained hovering treatments were higher than wingbeat frequencies of Anna's and rufous hummingbirds across treatments (Altshuler et. al., 2010; Altshuler & Dudley, 2003). Wingbeat frequencies were similar to those reported in other studies that used ruby-throated hummingbirds (Chai & Dudley, 1995; Chai & Dudley, 1997). The higher wingbeat frequencies of *Archilochus colubris* are not surprising because they are smaller and wingbeat frequency scales negatively with mass (Ellington, 1991).

It is generally agreed upon that birds fly with a fairly constant wingbeat frequency in order for muscles to contract at an optimal contraction frequency and velocity (Greenwalt, 1962; Tobalske et al., 1997; Pennycuick, 1992). However, wingbeat frequency increased significantly during air density reduction trials. Slight, but significant increases in wingbeat frequencies have also been observed in other air density reduction studies (Altshuler et. al., 2010, Chai & Dudley, 1997). However, when the lowest air density was omitted from the analysis the increase in wingbeat frequency was no longer significant, suggesting that this one data point was driving the trend (Figure 3B). Therefore, the wingbeat frequency only increased significantly at the lowest air density. Interestingly, wingbeat frequency did not vary significantly across sub-maximal load lifting trials. Rufous hummingbirds that lifted additional weights also increased stroke amplitude, rather than wingbeat frequency (Well, 1993). Therefore, it seems there are subtle differences in the way birds alter wingbeat kinematics to meet increased aerodynamic power output requirements that depend on the nature of the challenge or properties of the air. Birds that hover in less dense air may exhibit increased wingbeat frequencies as a result of decreased drag on the wings. Due to reduced drag on the wings, a given level of neural activation (and thus similar force production) may result in the wings accelerating more rapidly, and thus travelling a greater arc, through the less dense air. It is important to look at other wingbeat kinematic variables to see

if they differ between hovering in reduced air densities versus lifting sub-maximal loads. A difference in other kinematic variables would support the hypothesis that hummingbirds employ different strategies to increase aerodynamic power output based on the nature of the challenge. However, the significant increase in wingbeat frequency was driven by the lowest air density, which suggests that differences in the viscosity of ambient air versus heliox mixtures may only be significant at very low air densities.

Wingbeat frequency did not increase significantly as a function of load lifted. The wingbeat frequencies during maximal load lifting trials were between 54-58 Hz and wingbeat frequencies of unloaded hovering flight were between 51- 56 Hz, which is similar to values reported by Chai et al. (1997). Chai et. al. (1997), reported wingbeat frequencies of 57-58 Hz for maximally loaded male ruby-throated hummingbirds and wingbeat frequencies of 49 to 52 Hz during unloaded hovering flight. Although Chai et al., found the increase in wingbeat frequency to be significant, it is a modest increase. Therefore, it is not all that surprising that I did not find a significant increase in wingbeat frequency. Detecting a significant difference may be harder in ruby-throated hummingbirds since they seem to have relatively high wingbeat frequencies when hovering without any load attached. This suggests that they may have a lower capacity to increase wingbeat frequencies. A significant increase in wingbeat frequency between unloaded (140 to 160 Hz) and maximally-loaded trials (169 to 180 Hz) was observed in Anna's hummingbirds (Altshuler et al., 2010). Broad-tailed (*Selasphorus platycercus*) and rufous hummingbirds (*S. rufus*) that adopted higher frequency, lower stroke amplitude wingbeat kinematics when hovering at wide-diameter artificial flowers exhibited higher metabolic rates than those hovering at smaller flowers, suggesting that increasing wingbeat frequency was a more energetically expensive kinematic adjustment (Wells, 1993). The finding by Altshuler and Dudley (2003) that ruby-throated hummingbirds reduced wingbeat frequency when hovering in

normodense hypoxic air, while maintaining their stroke amplitude further supports the idea that frequency, rather than amplitude is the more energetically expensive kinematic feature to maintain. During maximal load lifting the same birds reached stroke amplitudes of 180 degrees and they increased wingbeat frequency (Altshuler and Dudley, 2003). In order to increase stroke amplitude to the geometrical limit of 180 degrees while simultaneously increasing wingbeat frequency, hummingbirds must possess the ability to increase muscle mechanical power output to a large extent. Altshuler & Dudley (2003) reported a two-fold increase in mechanical power output of muscles during maximal load lifting trials in ruby-throated hummingbirds. Such large increases in power output are expensive and hummingbird flight muscles are composed of fast-twitch oxidative glycolytic fibres. Therefore, the increases can only be borne by tapping into additional anaerobic energy stores. Hummingbirds ability for burst performance is probably supported by creatine kinase/phosphocreatine system and anaerobic glycolysis (Altshuler & Dudley, 2003). It has been reported that hummingbirds have high activities of creatine kinase in their primary flight muscles (Suarez et al., 1986). Phosphagen is depleted quickly, which explains why the birds descend to the bottom of the cage within 15 wingbeats of carrying the maximal load. The creatine kinase/phosphocreatine system provides a short-term reserve for energetically demanding activities, such as escaping from predators.

4.2 EMG Activation Patterns

As aerodynamic power output requirements increase muscle mechanical power output in the pectoralis and supracoracoideus also need to increase. The normalized EMG area increased for both muscles across increasing aerodynamic power output requirements. The increase in normalized EMG area suggests that the birds increase mechanical power output by increasing motor unit recruitment. The increase in EMG intensity due to increased aerodynamic power output requirements has been reported previously in hummingbirds, cockatiels, zebra finches,

and budgies (Altshuler et al., 2010; Tobalske et al., 2010; Hedrick et al., 2003; Ellerby & Askew, 2007; Tobalske & Dial, 1994). Although the results show that an increased force production is achieved at least partially through increased motor unit recruitment, conclusive statements cannot be made about other strategies that may be employed. An increase in muscle power output can be achieved by varying contraction frequency, percentage of shortening, timing and magnitude of neuromuscular activation or the strain trajectory. Budgies increase EMG intensity, muscle strain, and the amount of time spent shortening in order to increase aerodynamic power output (Ellerby & Askew, 2007). Cockatiels primarily increase muscle mechanical power output by varying the proportion of motor units that are activated. It is difficult to speculate on the strategies employed by hummingbirds because it is difficult to obtain *in vivo* measurements of strain changes on such small birds. There is only one published study that employed sonomicrometry to estimate strain trajectories for the pectoralis muscle of one rufous hummingbird across varying flight speeds (Tobalske et al., 2010). The strain in the pectoralis was only 10.8%, which is much lower than strains in other birds. More importantly, both total strain and strain rate did not exhibit any sort of a trend across varying flight speeds. However, they did find that there was a tendency for fractional lengthening to decrease and fraction shortening to increase as flight speed increased. The results from the Tobalske et al., (2010), suggests that hummingbirds may increase mechanical power output by increasing motor unit recruitment and strain trajectory, though it is unclear if similar patterns would apply to variation in power output during hovering. More definitive conclusions cannot be drawn, due to a sample size of one; therefore, further studies are necessary in order to decipher exactly how hummingbirds increase muscle power output.

In both the pectoralis and supracoracoideus muscles a discrete number of spikes per burst were observed. The number of spikes per burst is similar to other hummingbirds, and

substantially lower than observed in other birds (Altshuler et al., 2010; Tobalkse et al., 2010; Hedrick et al., 2003; Ellerby & Askew, 2007; Tobalske & Dial, 1994). The discrete number of spikes most likely results from the limited amount of time available for neuromuscular activation and deactivation when operating at high frequencies. In this study flight muscles in ruby-throated hummingbirds did not exhibit single spikes per burst like the pectoralis muscle of Anna's hummingbirds, even though their wingbeat frequencies are higher at all treatment levels (Altshuler et al., 2010). Therefore, while the number of spikes is limited such that it must occur within a restricted activation window it does not always result in EMGs consisting of single spikes per burst. EMG recordings of the tail shaker muscle of rattle snakes which operate at 90 hertz exhibit single spikes associated with each cycle of shortening. However, single spikes are not observed in all fast vertebrate muscles.

The number of spikes per burst only increased significantly during maximal load lifting assays. The increase in number of spikes per burst could have reflected increased spatial or temporal recruitment of motor units. Anna's hummingbirds also displayed a fairly constant number of spikes per burst during air density reduction trials and significant increases were only observed under maximal load lifting assays (Altshuler et al., 2010). Similarly, during sustained flight across varying flight speeds rufous hummingbirds did not change the number of spikes per burst. The results from previous studies along with our study suggest that hummingbirds only increase spike number in order to transiently generate maximum mechanical power output. This suggests that the increase in spike number reflects changes in motor unit activation patterns that cannot be aerobically sustained.

The EMG onset and offset of the pectoralis muscle preceded the downstroke. The activation of the pectoralis occurs about halfway into the upstroke, when the muscle is lengthening. This, most likely results in the force developing when the pectoralis is slightly

lengthened which enhances the rate of force rise and the peak force production (Biewener, 2011; Taube, 2012). Similarly the EMG onset and offset of the supracoracoideus occurred prior to the upstroke. This pattern of muscle offset prior to shortening has been found in this study in ruby-throated hummingbirds and previously in Anna's (Altshuler et al., 2010) and rufous hummingbirds (Tobalske et al., 2010). These results support the idea that this pattern of muscle offset prior to shortening is a general feature of muscles operating faster than 40 Hz.

4.3 Comparison of Pectoralis and Supracoracoideus

The pectoralis and the supracoracoideus are the primary flight muscles that generate almost all of the mechanical power output required for hovering (Biewener, 2011; Tobalske, 2010). The pectoralis and supracoracoideus have anatomical differences, but they have similar functions (Tobalske, 2011; Greenwalt, 1962). Given these anatomical and functional similarities, I hypothesized that they would exhibit similar variation in EMG activation patterns. This is the first study where EMGs of both muscles were collected during hovering under varying aerodynamic power requirements. The only other study that looked at both muscles simultaneously was conducted by Tobalske et al., (2010), on rufous hummingbirds during forward flight. Rufous hummingbirds were placed in a wind tunnel and trained to fly across a range of speeds. Although, hovering does differ from forward flight, it is the only study in hummingbirds available in the literature with which comparison of results is possible. In this study, the normalized EMG area did not differ between the two muscles; therefore, the activation of both muscles were similar in magnitude across varying aerodynamic power outputs compared to maximum observed activation intensities. Tobalske et al. (2010), found a strong correlation between relative EMG amplitudes between the two muscles across varying flight speeds, which also suggests that the relative changes in the activation does not differ between muscles across varying flight speeds. However, the number of spikes and EMG onset did differ between the two

muscles. The supracoracoideus consistently exhibited a greater number of spikes than the pectoralis during air density reduction trials, sub-maximal load lifting trials and maximal load lifting trials. This was also observed in rufous hummingbirds during forward flight; where the pectoralis exhibited 2.9 ± 0.8 spikes per burst while the supracoracoideus displayed 3.8 ± 0.8 spikes per burst (Tobalske et al., 2010). In my study, the EMG onset of the supracoracoideus prior to upstroke occurred earlier than the EMG onset of the pectoralis prior to the downstroke. Similar differences in EMG onset of the two muscles were observed in rufous hummingbirds (Tobalske et al., 2010). The earlier onset of activity in the supracoracoideus prior to the upstroke also suggests that it is activated earlier with respect to the beginning of muscle shortening (i.e. the change in muscle length as measured from point of origin to point of insertion). Although superficially suggested by the EMG activity and kinematics, it is unlikely that the electrical activity and muscle force development are completely out of phase. The beginning of wrist elevation and depression almost certainly do not represent the onset of all kinematic or biomechanical effects of the pectoralis and supracoracoideus, because, as in other birds, it is likely that these muscles do more than elevate and depress the wing. The forces from the pectoralis and supracoracoideus are used to decelerate the wing, stabilize the shoulder and effect long-axis rotation of the humerus at transitions between half strokes (Poore et al., 1997; Tobalske & Biewener, 2008). Furthermore, at least during hovering, hummingbirds produce rotational circulation and lift at wing turnaround using long-axis pronation and supination of the wing (Warrick et al., 2009). The differences in the EMG activation patterns of the two primary flight muscles may stem from anatomical differences. The horizontal stroke plane and relative symmetry of the hovering upstroke and downstroke in hummingbirds suggests that the mechanical power required to accelerate the wing through each half stroke is more similar than in other birds. The increased number of spikes per burst in the supracoracoideus in comparison to

the pectoralis may represent a strategy employed to generate greater power per unit muscle mass in order to compensate for its smaller size. The pectoralis is about twice the size of the supracoracoideus (Greenwalt, 1962). Since fibre type is homogenous in both muscles (Welch and Altshuler, 2009) methods for varying power output are limited to variation in spatial or temporal recruitment of motor units. With fewer fibres available, it is possible greater temporal recruitment plays a role in permitting power output in the supracoracoideus to better match power output in the pectoralis, and the consistent occurrence of more spikes per burst in the supracoracoideus supports this hypothesis. The supracoracoideus also has a very long tendon attached to it, unlike the pectoralis (Biewener, 2011). The earlier onset of the supracoracoideus prior to the upstroke may be the result of having to shorten fibres and thus lengthen the tendon before causing any changes to the overall muscle length. Generally, the long, thin tendons attached to the belly of pennate distal hindlimb muscles of vertebrates with short fibres, are highly compliant (Roberts, 2002). Muscles with tendons that are highly compliant expend a large fraction of their shortening capacity stretching the tendon rather than causing skeletal movements directly. Tendons can uncouple the muscle activation and shortening from the movement of the body. Muscles with long fibres and short tendons, tend to have tendons with low compliance. In these muscles the length changes occur primarily in the contractile element (Roberts, 2002). Therefore, the supracoracoideus may have to be activated earlier prior to the upstroke in order to lengthen its long tendon before shortening and starting the upstroke.

4.4 Comparison of Air Density Reduction Trials Versus Load Lifting Assays

Air density reduction trials have often been employed to study how animals increase power output (Altshuler et. al., 2010; Altshuler & Dudley, 2003; Chai & Dudley, 1996; Chai & Dudley, 1995). However, the possible passive effect of the reduced air density on drag and thus wing movement has been overlooked. A comparison of air density trials and load lifting trials

showed that there was no difference in EMG activation patterns between the two experiment types. For the same normalized EMG area, the stroke amplitude and wingbeat frequency did not differ between the two experimental types. Since the relationship between EMG intensity and kinematics were similar between treatment types, this suggests that wingbeat frequency in air density trials did not change solely as a result of decreased drag on the wings.

5. Overall Conclusions

The high wingbeat frequencies of ruby-throated hummingbirds limit the amount of time available for the activation and deactivation of primary flight muscles. The limited time window has resulted in motor unit activation being more synchronized, which has led to simpler, readily interpretable EMG traces. Consistent with previous studies, the activation and deactivation of the pectoralis and supracoracoideus muscles occurred prior to the start of the downstroke and the upstroke, respectively. As hypothesized, normalized EMG area did increase as aerodynamic power requirements increased and it was the best predictor of muscle mechanical power output and wingbeat kinematics. The increase in EMG area represents spatial and/or temporal recruitment of motor units. Across air density reduction trials and sub-maximal load lifting trials EMG patterns were not different; however, the results from the wingbeat kinematics did differ slightly. The results suggest that the reduced drag at very low air densities may permit increased wingbeat frequency, independent of increases in intensity of neuromuscular activation. Contrary to the hypothesis that activation patterns would be similar between the pectoralis and supracoracoideus, the timing of activation and number of spikes per EMG burst were consistently different between the two muscles, likely reflecting differences in muscle morphology.

Hummingbirds increased mechanical power output in both flight muscles by increasing the recruitment of motor units regardless of whether the birds were lifting additional loads or hovering in less dense air. The increase is mechanical power output translated to an increase in stroke amplitude, except at the lowest air density, in which wingbeat frequency also increased. The pectoralis and supracoracoideus differ morphologically. They exhibited similar relative increases in motor unit recruitment as mechanical power requirements increased, but differences in other EMG measurements were observed. Therefore, morphology of a muscle and activation patterns are linked to one another.

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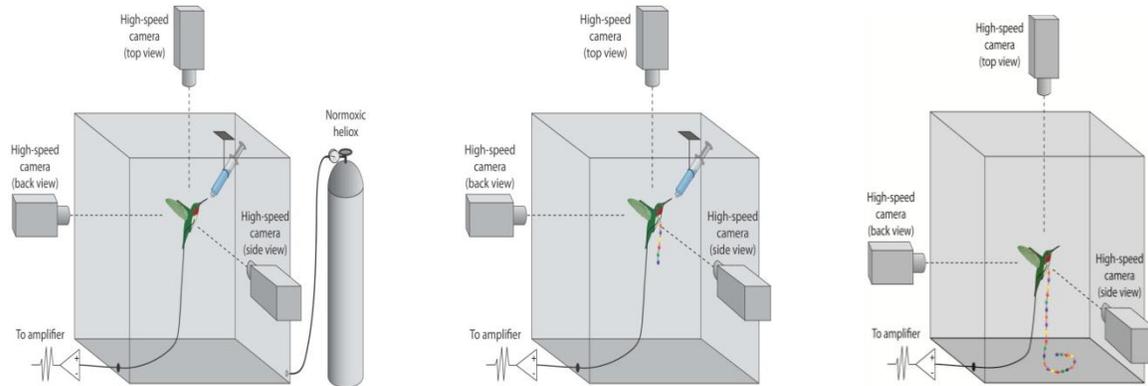
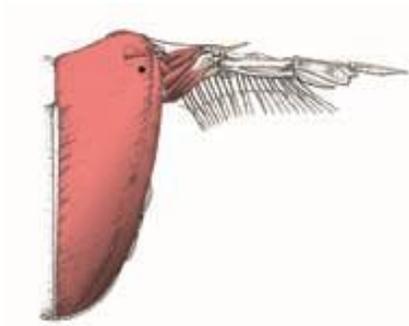


Figure 1. Experimental set-up for all *in vivo* recordings. A) Air density reduction trials: air density was decreased by filling the chamber with heliox. B) Sub-maximal load lifting trials: the birds fed while lifting 0.25, 0.5, and 0.75 g harnesses 3) Maximal load lifting trials: the birds were placed at the bottom of the arena with a harness around their neck. The birds transiently lifted the maximal load as they tried to ascend. Ambient air trials were conducted using an identical setup as in the submaximal load lifting trials except birds hovered while lifting no load at all.

A



B

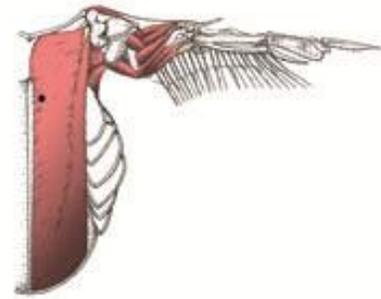


Figure 2. An illustration of the musculoskeletal anatomy of small hummingbirds. Black dots indicate the position of electrode placement in (A) the pectoralis and (B) the supracoracoideus muscle. Note the illustration in B is identical to that in A except that the pectoralis has been removed to show the supracoracoideus muscle, which lies deep to it.

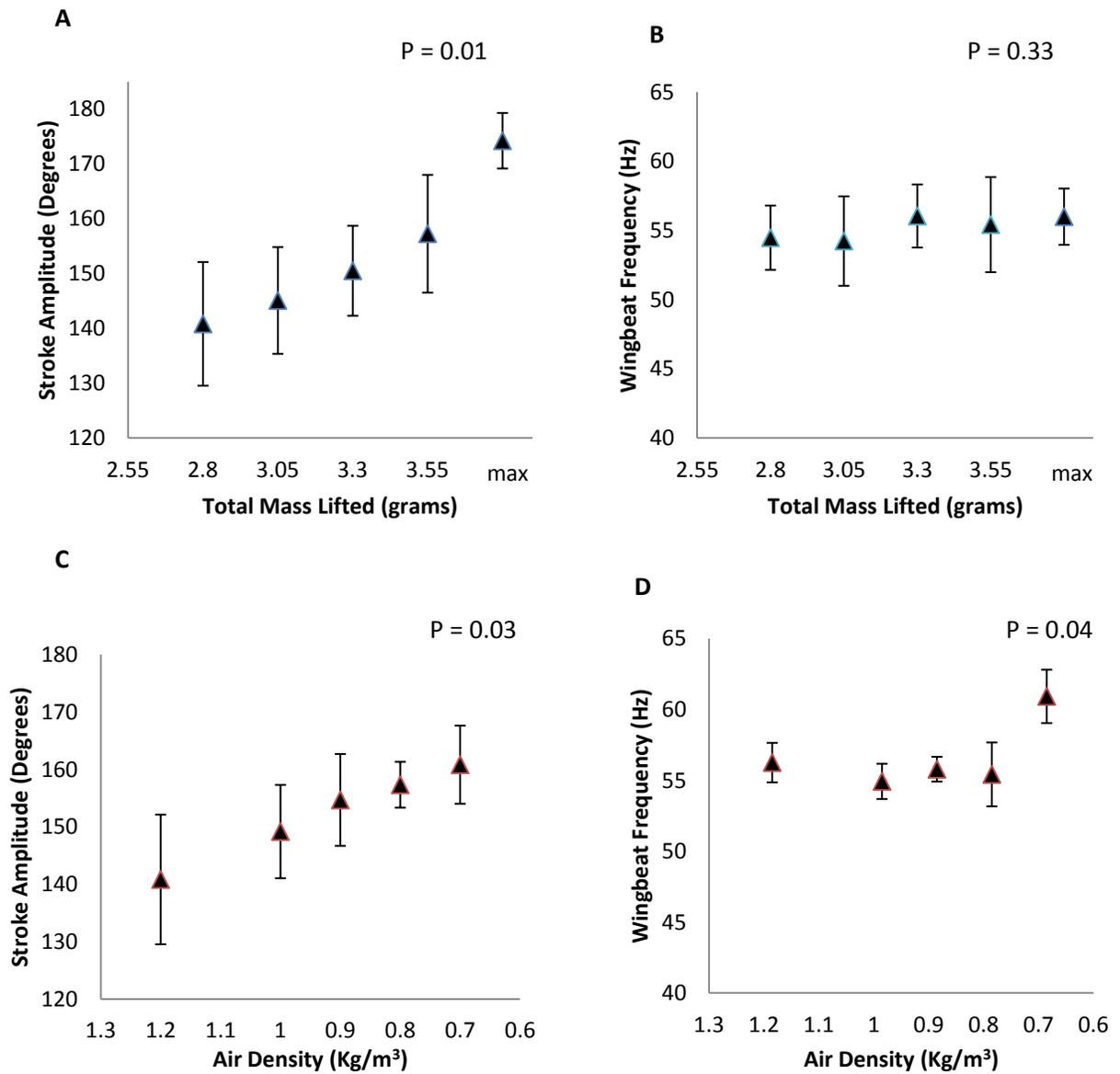


Figure 3. Stroke amplitude (A, C) and wingbeat frequency (B, D) in relation to air density (A, B) or total mass lifted (C, D) for four individual ruby-throated hummingbirds (*Archilochus colubris*) while hovering. Max on the x-axis represents the maximal load lifting assay. Symbols represent mean (\pm s.d.) of the four hummingbirds.

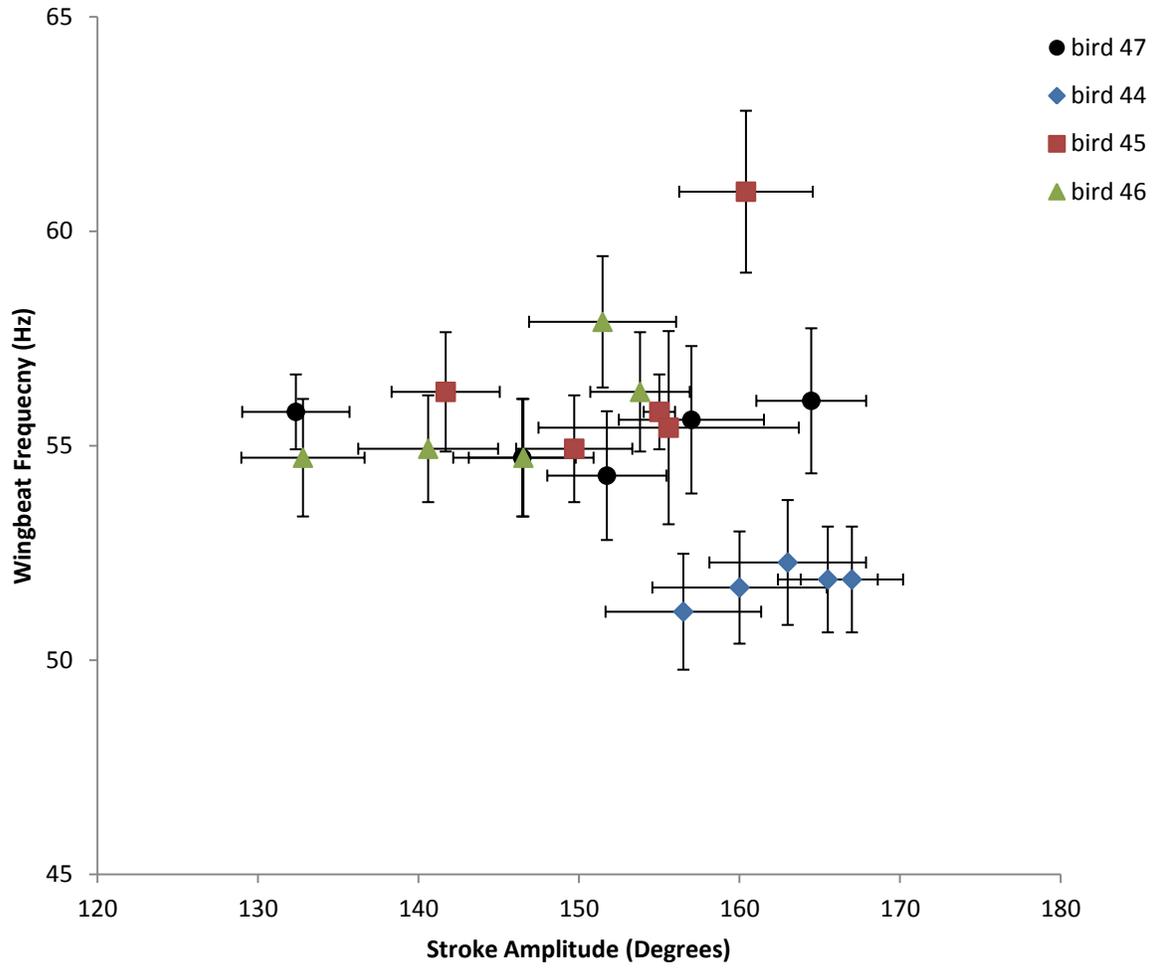


Figure 5. Individual variation in wingbeat frequency (wf) and stroke amplitude (sa) across decreasing air densities. Symbols represent mean (\pm s.d.) of individual hummingbirds.

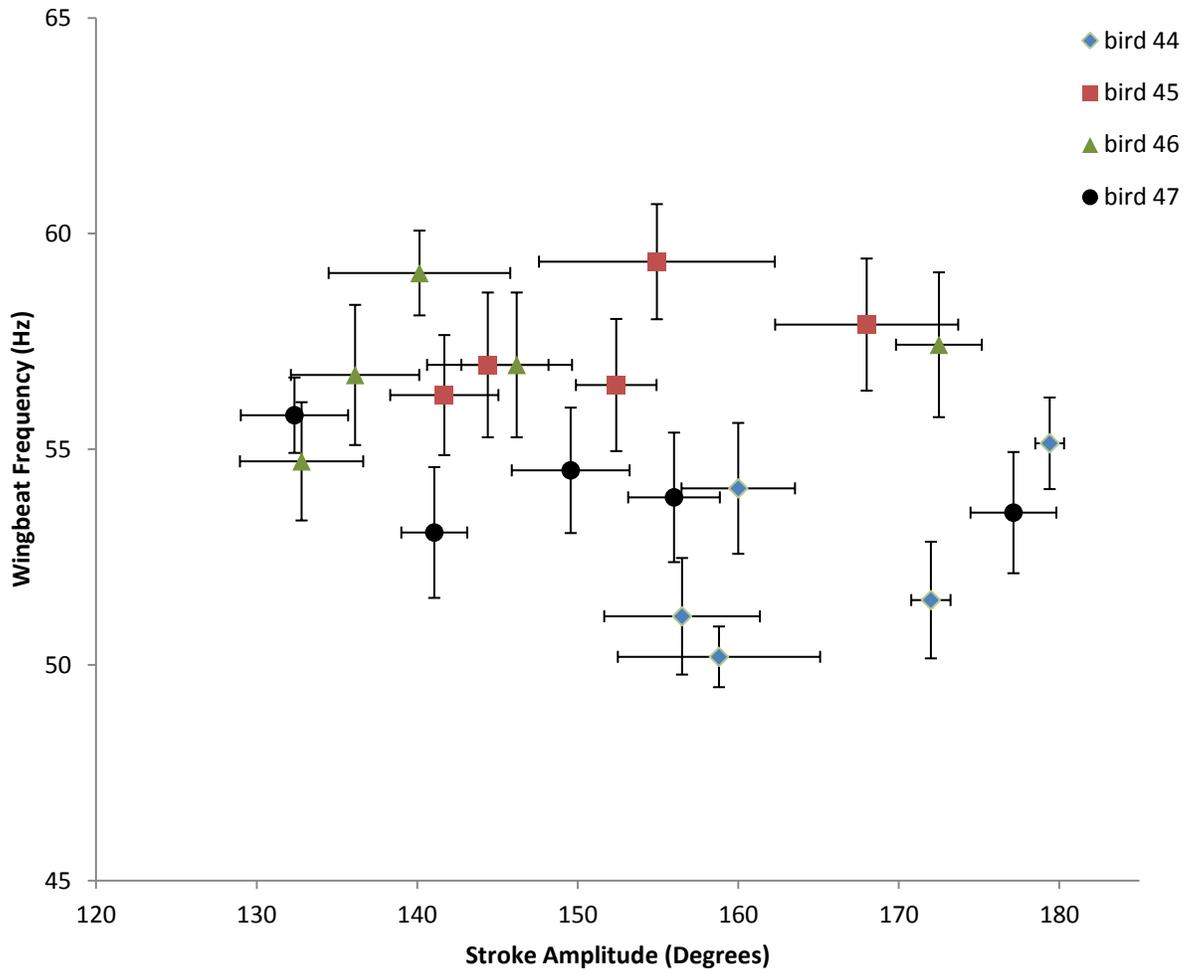


Figure 5. Individual variation in wingbeat frequency (wf) and stroke amplitude (sa) across increasing loads. Symbols represent mean (\pm s.d.) of individual hummingbirds.

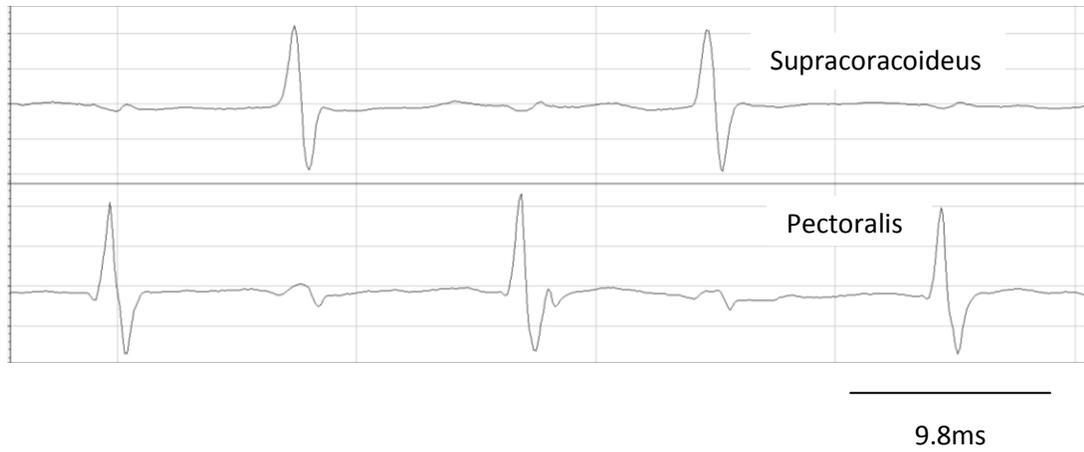


Figure 6. Sample EMG recording of both flight muscles while the bird was hovering in ambient air. The first channel is an EMG trace of the supracoracoideus muscle and the second channel is an EMG trace from the pectoralis.

Load Lifting Trials

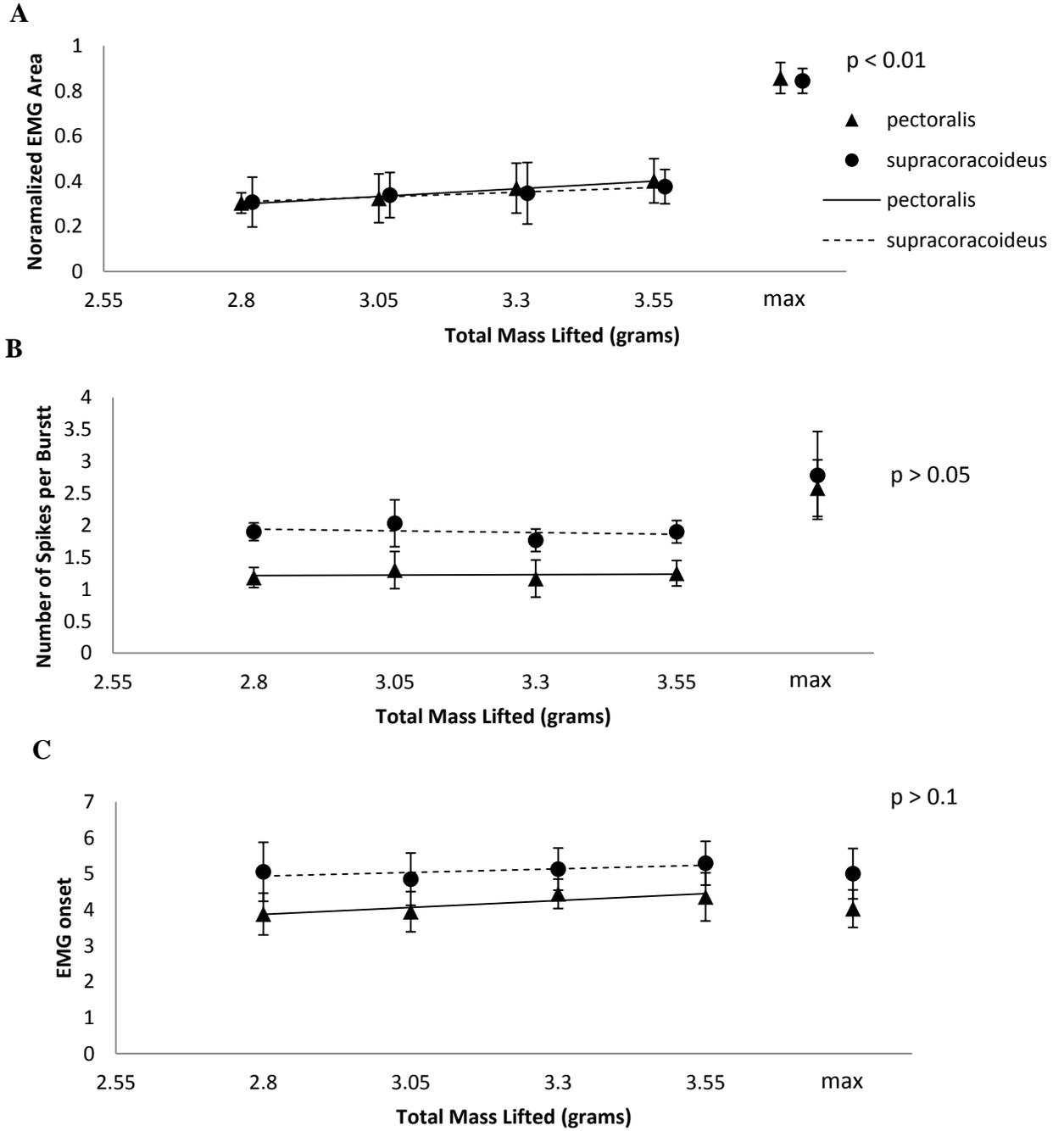


Figure 7. EMG variables, normalized EMG intensity (A), number of spikes per burst (B), and EMG onset (C) in relation to decreasing air densities for the supracoracoideus and pectoralis muscles. Max on the x-axis represents maximal load lifting trials. The symbols represent means (\pm s.d.) of individual hummingbirds. Normalized EMG area of both muscles increased significantly as total mass lifted increased.

Air Density Reduction Trials

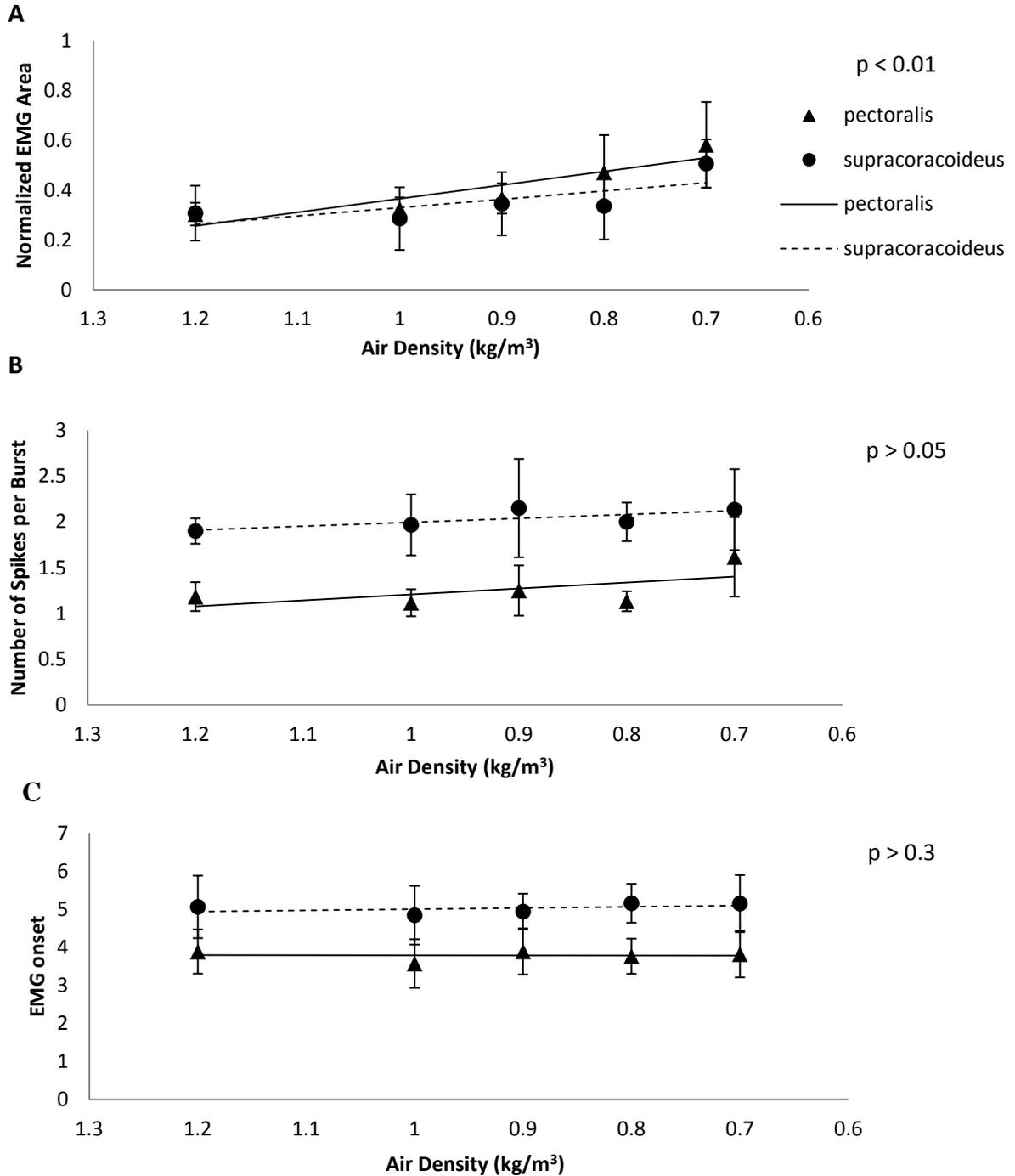


Figure 8. EMG variables, normalized EMG intensity (A), number of spikes per burst (B) and EMG onset (C) in relation to decreasing air densities for the supracoracoideus and pectoralis muscles. The symbols represent means (\pm s.d.) of individual hummingbirds. Normalized EMG area of both muscles increased significantly as air density decreased.

Load Lifting Assays

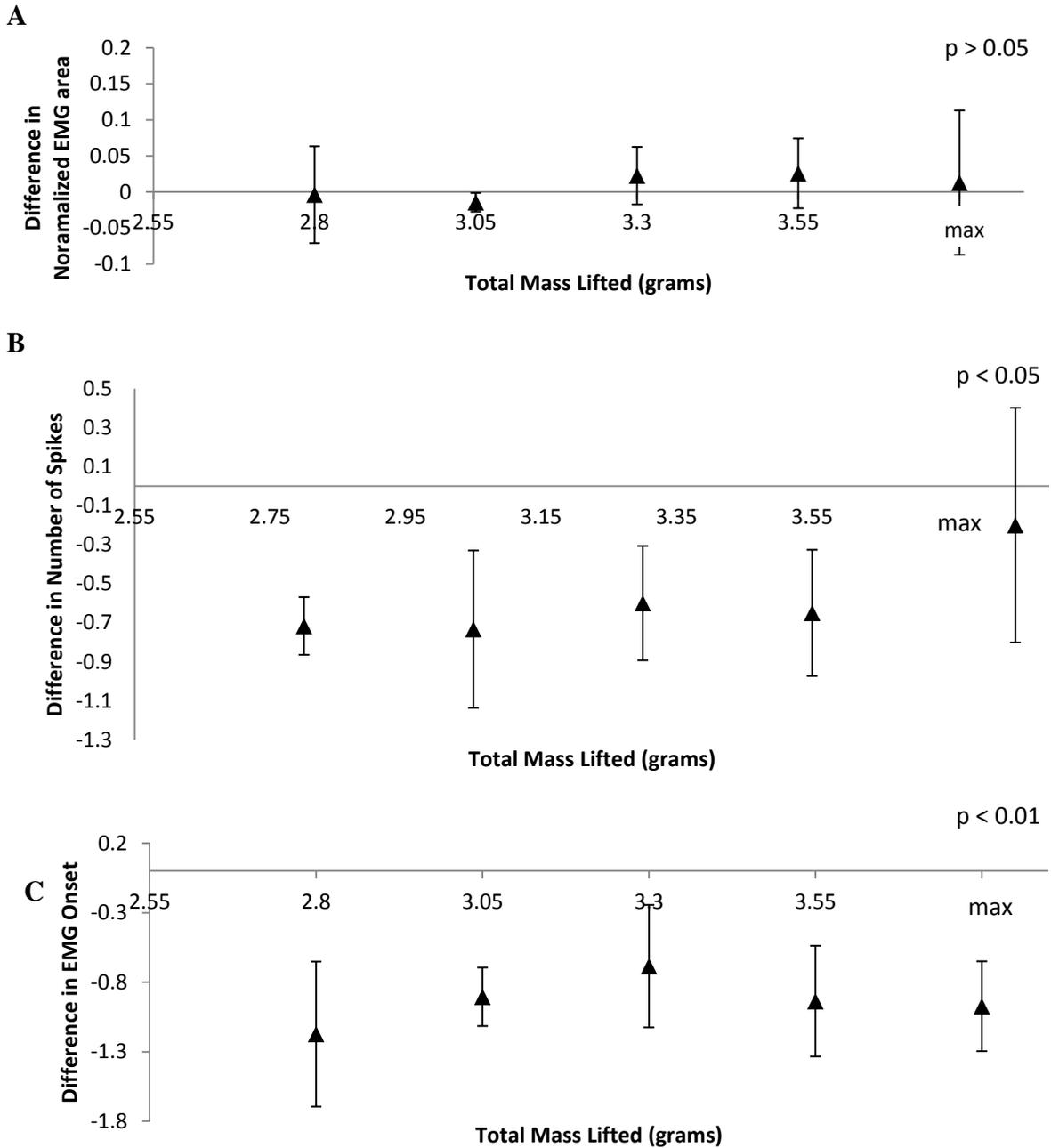


Figure 9. Comparison of the pectoralis and supracoracoideus in the regulation of EMG variables, normalized EMG intensity (A), number of spikes per burst (B), and EMG onset (C) in relation to total mass lifted. The symbols represent the average difference between the means of the pectoralis and supracoracoideus values of the four birds (pectoralis minus the supracoracoideus). Positive values indicate that the values for the pectoralis were larger and negative values indicate that values from the supracoracoideus were larger. The number of spikes per burst and the onset of the EMG were significantly different between the pectoralis and the supracoracoideus muscles across load lifting trials.

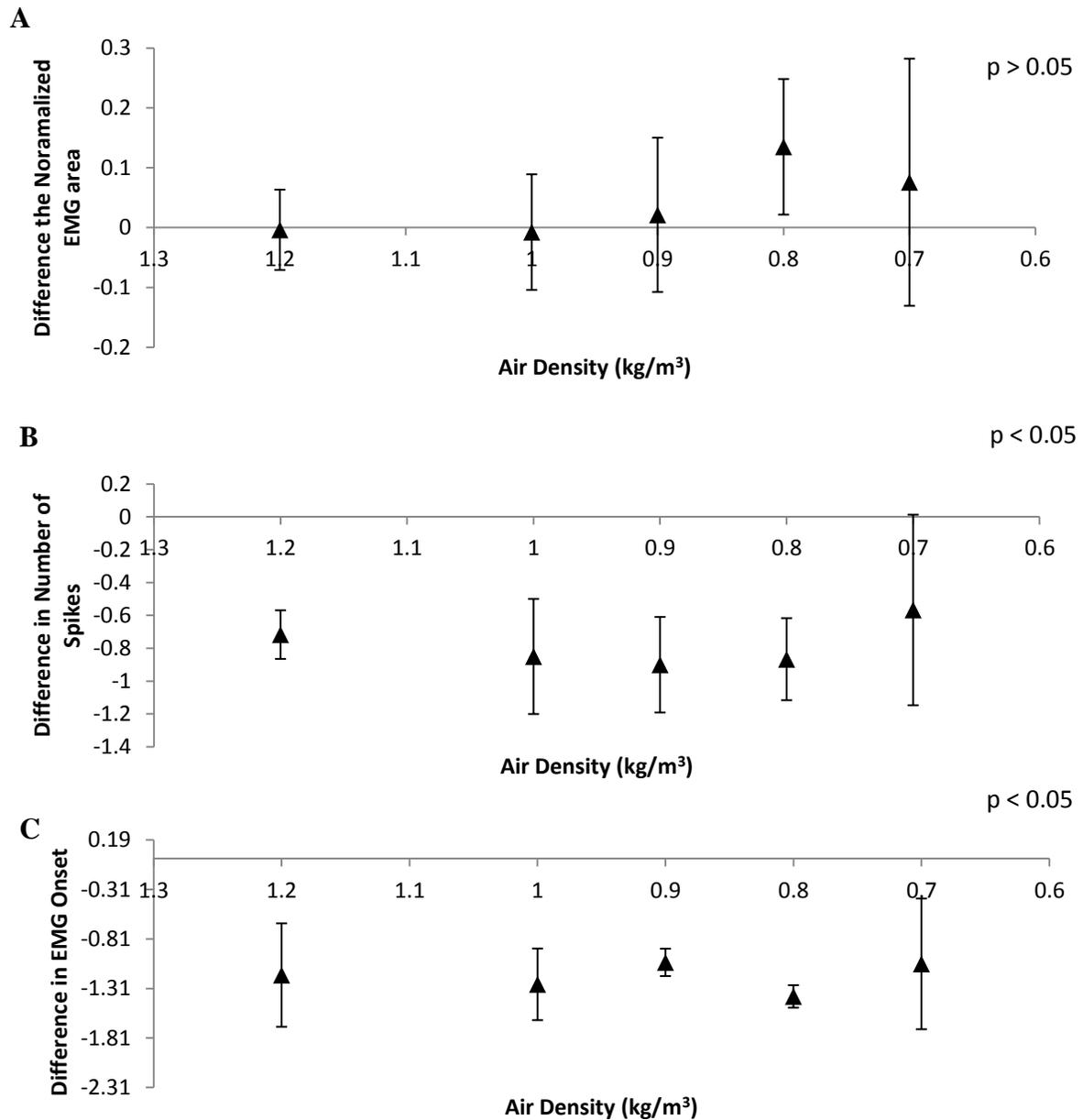


Figure 10. Comparison of the pectoralis and supracoracoideus muscles in the regulation of EMG variables, normalized EMG intensity (A), number of spikes per burst (B), and EMG onset (C) in relation to air density. The symbols represent the average difference between the means of the pectoralis and supracoracoideus values of the four birds (pectoralis minus the supracoracoideus). Positive values indicate that the values for the pectoralis were larger and negative values indicate that values from the supracoracoideus were larger. The number of spikes per burst and the onset of the EMG were significantly different between the pectoralis and the supracoracoideus muscles across air density trials.

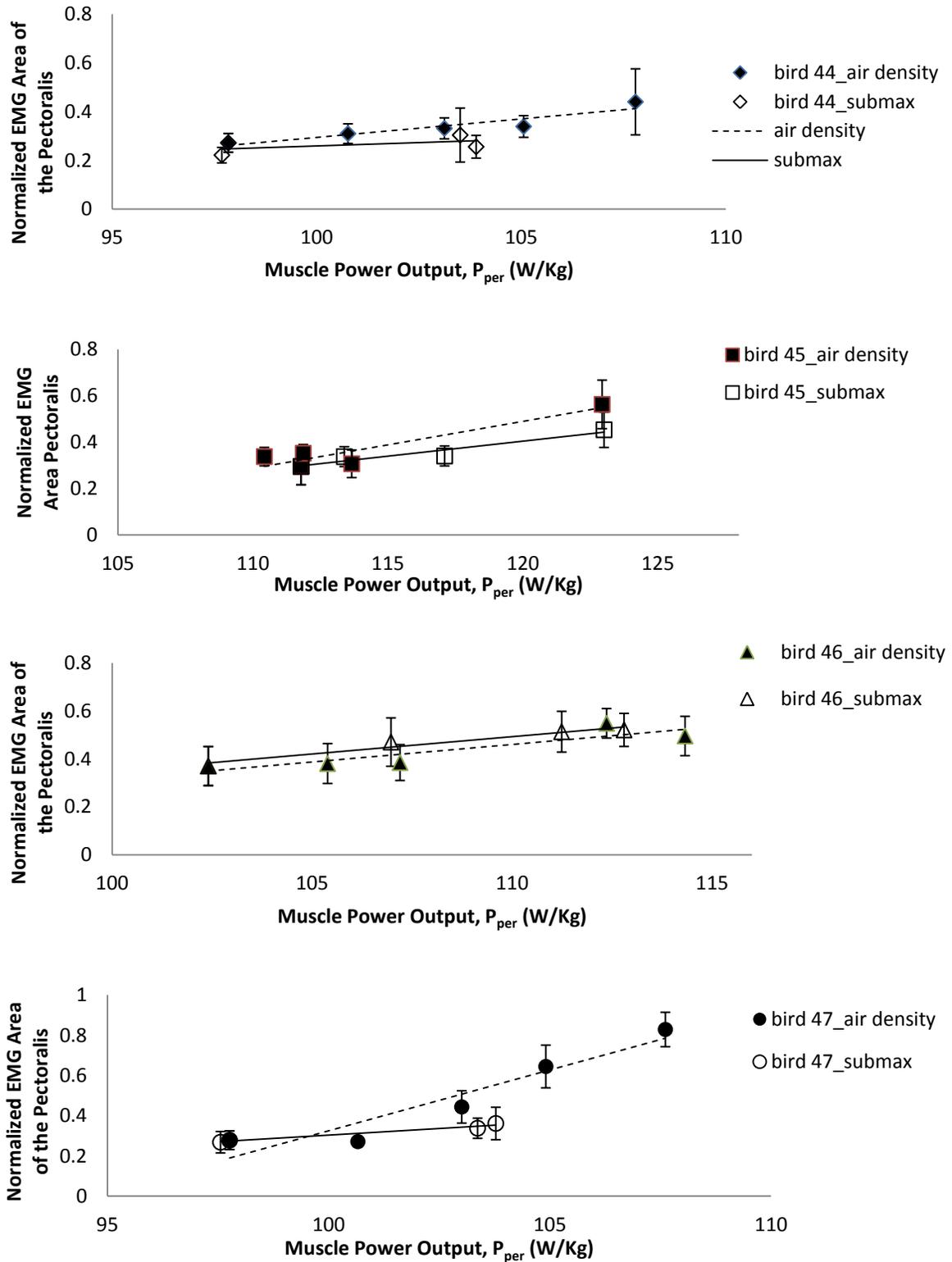


Figure 11. Normalized EMG area of the pectoralis muscle in relation to muscle power output across air density reduction trials and sub-maximal load lifting assays. Symbols represent means (\pm s.d.) for individual hummingbirds. There was a significant effect of power output on normalized EMG area ($p = 0.002$). There was not a significant effect of experiment type ($p = 0.84$) or the interaction of power output and experiment type ($p=0.76$) on normalized EMG area.

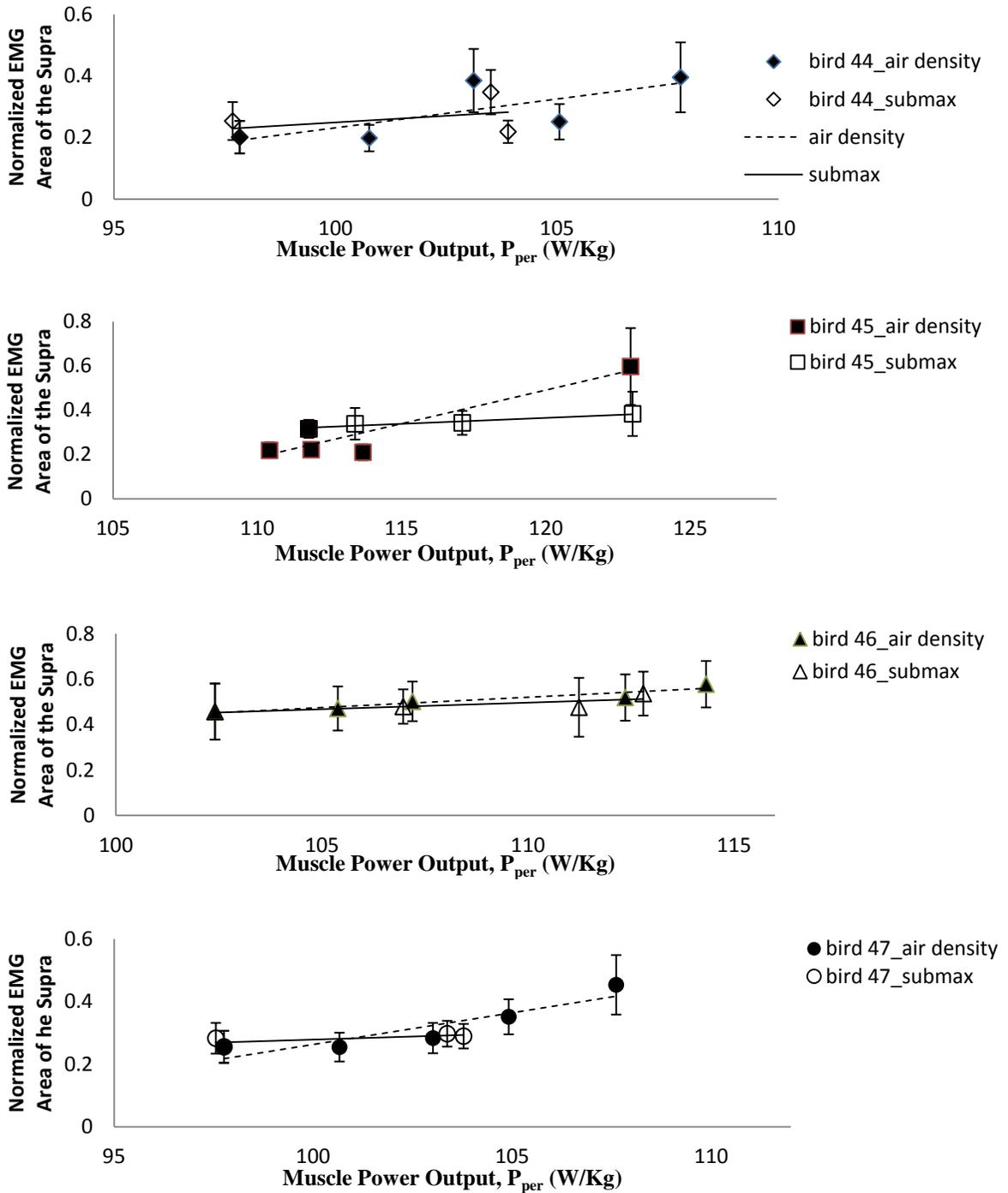


Figure 12. Normalized EMG area of the supracoracoideus muscle in relation to muscle power output across air density reduction trials and sub-maximal load lifting assays. Symbols represent means (\pm s.d.) for individual hummingbirds. There was a significant effect of power output on normalized EMG area ($p < 0.001$). There was not a significant effect of experiment type ($p = 0.23$) or the interaction of power output and experiment type ($p = 0.22$) on normalized EMG area.

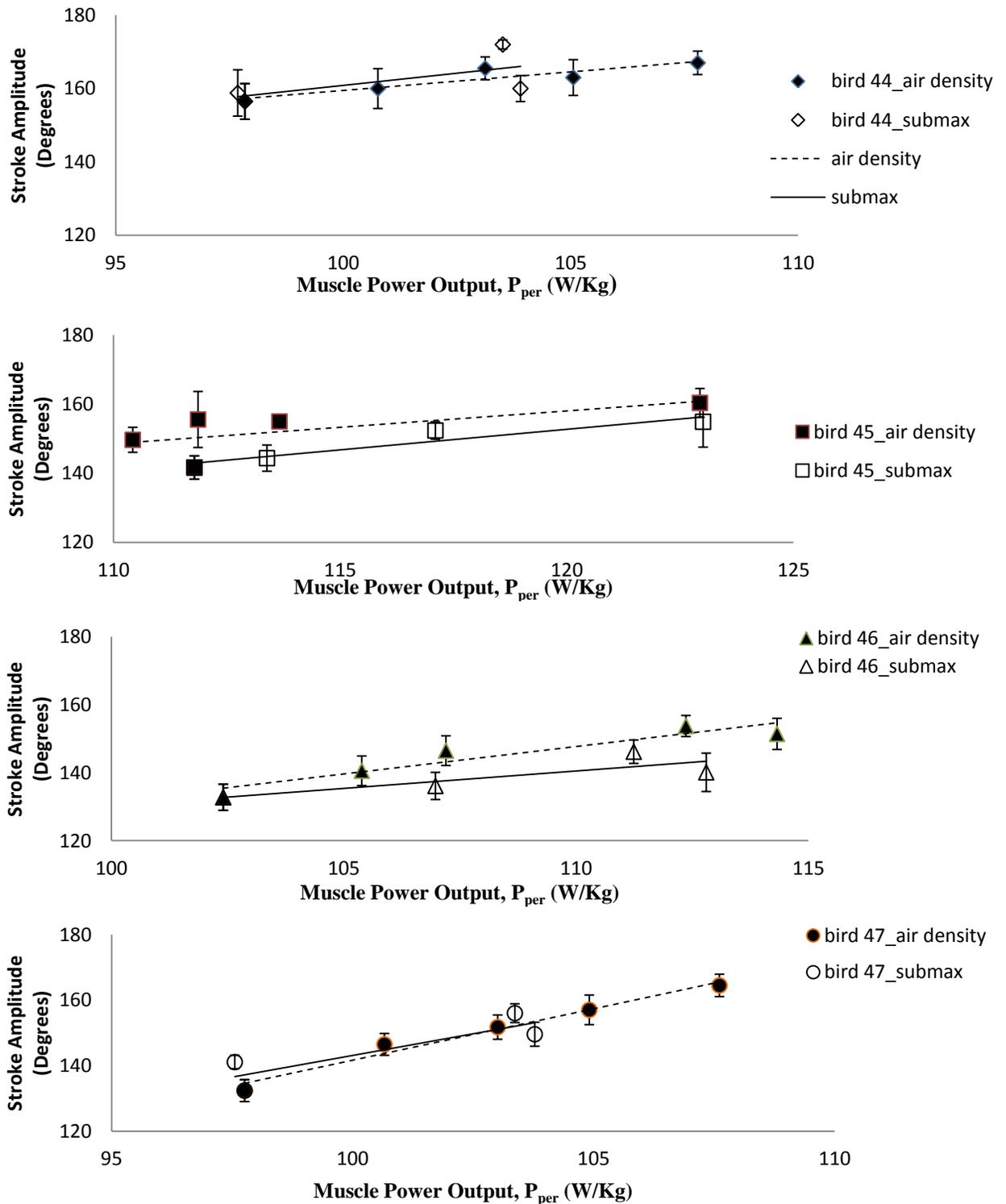


Figure 13. Stroke amplitude in relation to muscle power output under cross density reduction trials and load lifting assays. Symbols represent means (\pm s.d.). There was no significant difference between the trials types. There was a significant effect of power output on stroke amplitude ($p = 0.001$). There was not a significant effect of experiment type ($p = 0.83$) or the interaction of power output and experiment type ($p=0.75$) on stroke amplitude.

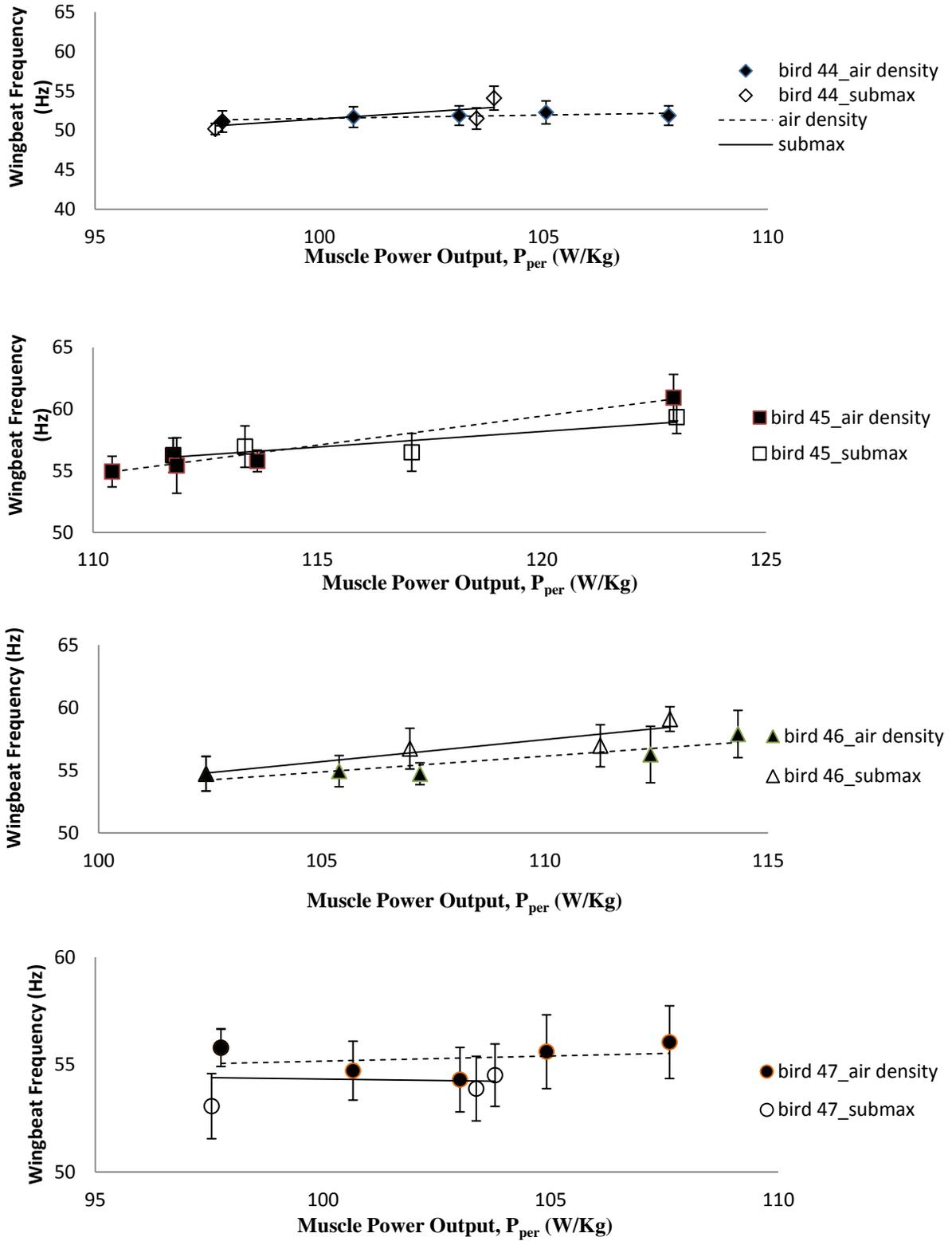


Figure 14. Wingbeat frequency in relation to muscle power output under across density reduction trials and load lifting assays. Symbols represent means (\pm s.d.). There was no significant difference between the trials types. There was a significant effect of power output on wingbeat frequency ($p < 0.001$). There was not a significant effect of experiment type ($p = 0.93$) or the interaction of power output and experiment type ($p=0.88$) on wingbeat frequency.

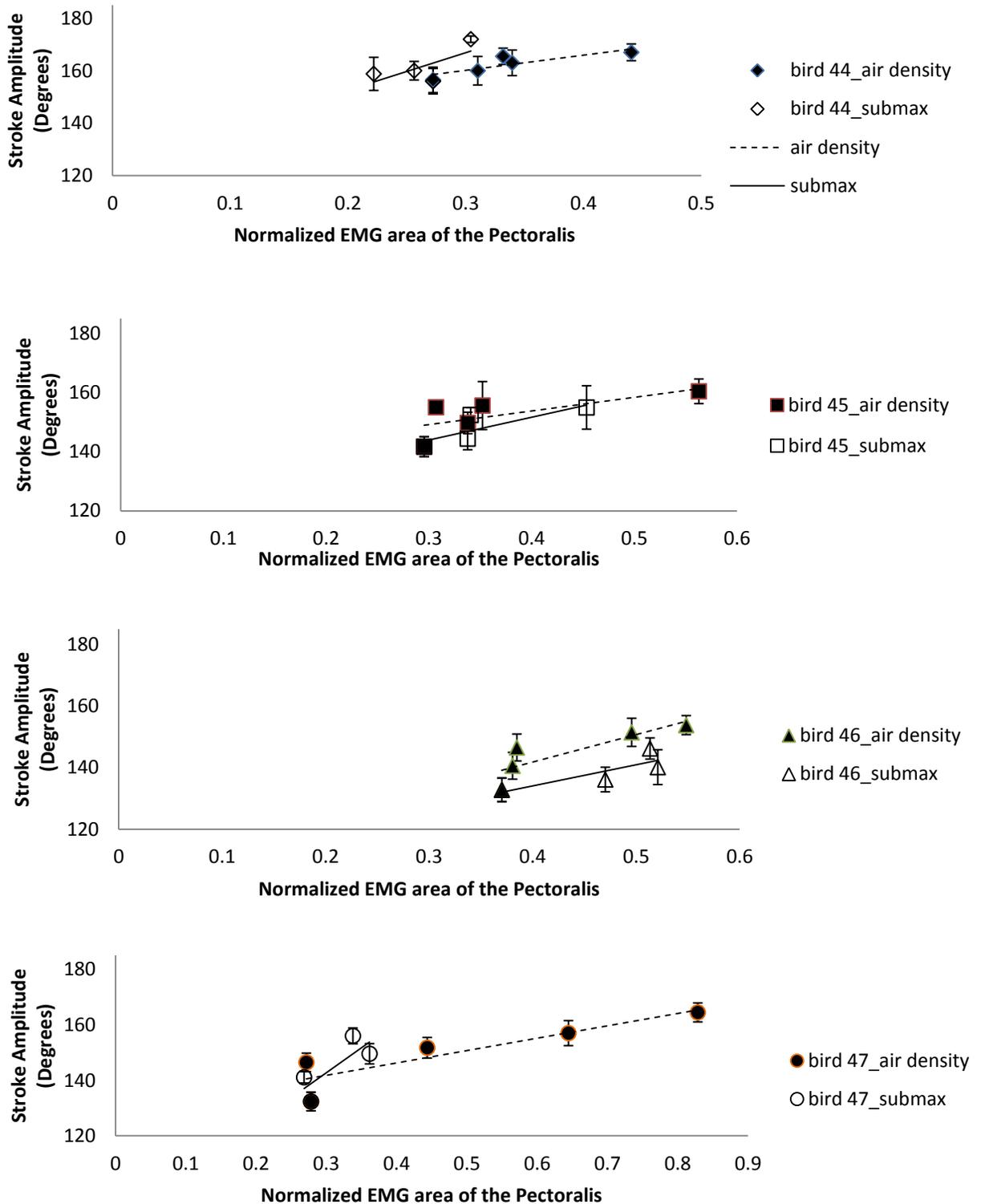


Figure 15. Stroke amplitude in relation to normalized EMG area of the pectoralis muscle across air density reduction trials and load lifting assays. Symbols represent means (\pm s.d.). There was no significant difference between the trials types. There was a significant effect normalized EMG area on stroke amplitude ($p = 0.001$). There was not a significant effect of experiment type ($p = 0.46$) or the interaction of normalized EMG area and experiment type ($p = 0.34$) on stroke amplitude.

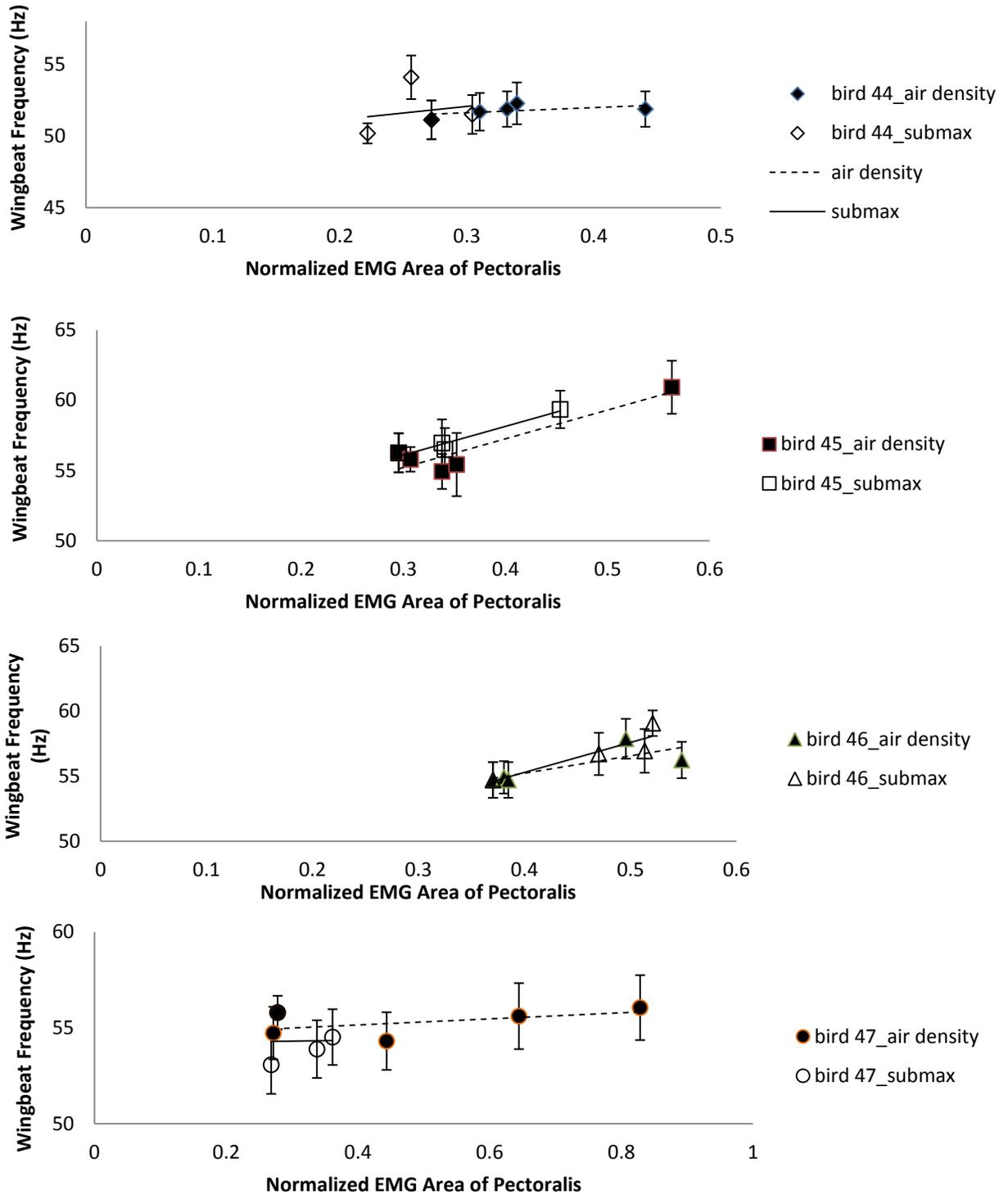


Figure 16. Wingbeat frequency in relation to normalized EMG area of the pectoralis muscle across air density reduction trials and load lifting assays. Symbols represent means (\pm s.d.). There was a significant effect normalized EMG area on wingbeat frequency ($p = 0.008$). There was not a significant effect of experiment type ($p = 0.19$) or the interaction of normalized EMG area and experiment type ($p = 0.09$) on wingbeat frequency.

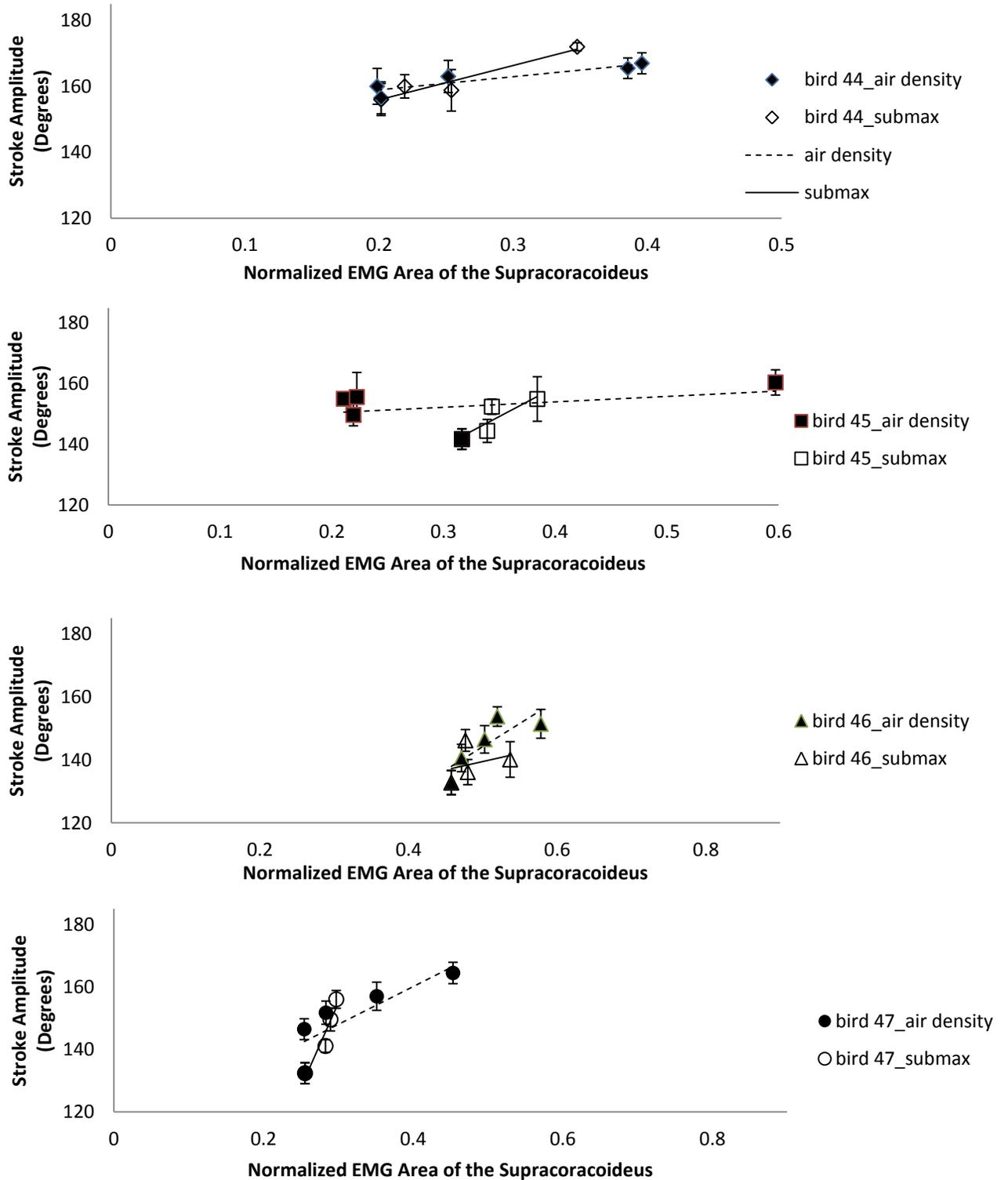


Figure 17. Stroke amplitude in relation to normalized EMG area of the supracoracoideus muscle across air density reduction trials and load lifting assays. Symbols represent means (\pm s.d.). There was no significant difference between the trials types. There was a significant effect normalized EMG area on stroke amplitude ($p = 0.002$). There was not a significant effect of experiment type ($p = 0.94$) or the interaction of normalized EMG area and experiment type ($p = 0.61$) on stroke amplitude.

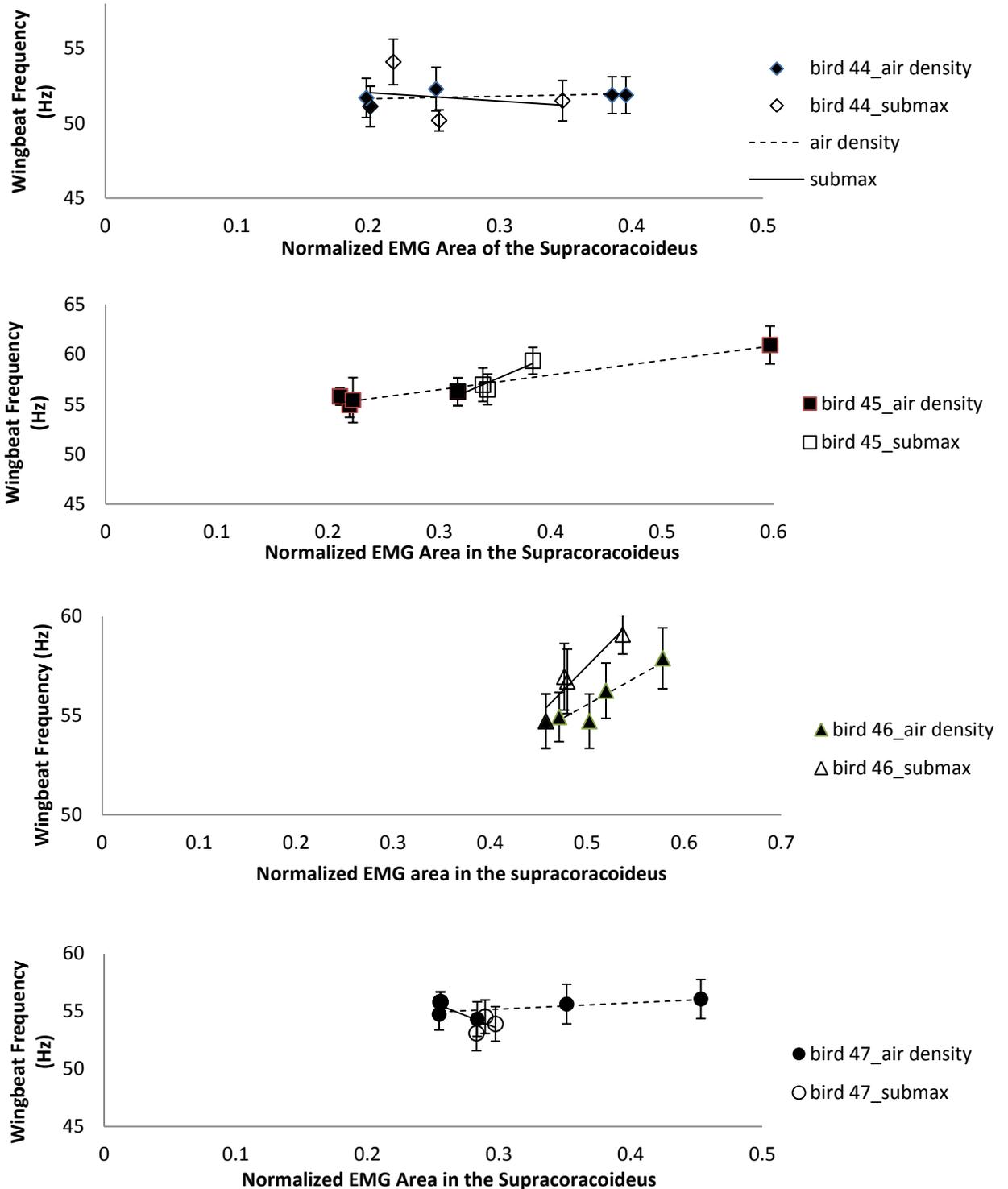


Figure 18. Wingbeat frequency in relation to normalized EMG area of the supracoracoideus muscle across air density reduction trials and load lifting assays. Symbols represent means (\pm s.d.). There was a significant effect normalized EMG area on wingbeat frequency ($p < 0.001$). There was not a significant effect of experiment type ($p = 0.35$) or the interaction of normalized EMG area and experiment type ($p = 0.22$) on wingbeat frequency.