Work loop dynamics of the pigeon (Columba livia) humerotriceps and its potential role for active wing morphing

by

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B.Sc., University of Calgary, 2015

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies

(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

September 2017

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Abstract

Avian wings change shape during the flapping cycle due to the activity of a network of intrinsic wing muscles. Wing control is believed to be the key feature allowing birds to maneuver safely through different environments. One control aspect is elbow joint motion, which relates to wing folding for the upstroke and re-expansion for the downstroke. Muscle anatomy suggests that if the muscles are actuating then the biceps flex the elbow, and the two heads of the triceps, the humerotriceps and scapulotriceps, extend the elbow. This set of antagonist muscles could thus actively modulate wing shape by regulating elbow joint angle. Control of the elbow joint angle remains uncertain as motor elements can have diverse functions such as actuators, brakes, springs, and struts, where specific roles and their magnitudes depend on when muscles are activated in the contractile cycle. The wing muscles best studied during flight are the elbow muscles of the pigeon (Columba livia). In vivo studies during different flight modes revealed variation in strain profile, activation timing and duration, and in contractile cycle frequency of the humerotriceps. This variation suggests that the pigeon humerotriceps may alter wing shape in diverse ways. To test this hypothesis, I developed an in situ work loop technique to measure the performance of the pigeon humerotriceps. My experiments tested how activation duration and contractile cycle frequency influenced muscle work and power across the full range of activation onset times. I found that the humerotriceps generated net positive power over a narrow range of activation times. The humerotriceps produced predominantly net negative power, likely due to relatively long activation durations, indicating that it absorbs work, but the work loop shapes also suggest varying degrees of elasticity and resistance. I was unable to examine the effects of variation in strain profile because current work loop technology does not allow for this. Nonetheless, these results, when combined with previous in vivo studies, show that the humerotriceps can dynamically shift among roles of brake, spring, and strut, based on activation properties that vary with flight mode.
Lay Summary

Birds are believed to perform different flight behaviours by actively modulating their wing shape using an extensive network of wing muscles. Elbow joint motion strongly contributes to an important behaviour: folding the wing for the upstroke and re-expansion for the downstroke. Muscle anatomy suggests that the two triceps muscles, the humerotriceps and scapulotriceps, control elbow extension. How elbow joint motion is controlled is unknown because muscle function is diverse. A muscle can generate work to actuate motion, or absorb it and decelerate motion, muscles can even serve as springs or struts. For my study, I wanted to gain insight into the function of the humerotriceps muscle by measuring its work and power output under different conditions in pigeons. Net work and power output from the humerotriceps was predominantly negative, regardless of the conditions, suggesting that this muscle serves primarily as a brake, helping to slow elbow flexion and wing folding.
Preface

All of the work presented within this manuscript were conducted in the Flight Laboratory at the University of British Columbia, under the guidance and supervision of Dr. D. Altshuler. All of the text within this manuscript are original and unpublished material. The experimental research reported in Chapter 2 was covered by UBC Animal Care Certificate number A15-0116.

For the research outlined in Chapter 2, I adapted and refined the experimental design and data collection procedure that were originally conceptualized by Dr. J. Bahlman and Dr. D. Altshuler. I followed the experimental protocols that were previously used by Dr. J Bahlman within a similar study, but for a different muscle, and within a different species. I adjusted these protocols to fit the needs of my research using my own ideas, and insight contributed by Dr. D. Altshuler, Dr. J. Bahlman, and Dr. D. Syme.

I was responsible for collecting the data contained within Chapter 2, which I achieved with some assistance from my colleagues: Dr. J. Bahlman, Dr. V. Baliga, and J. Wong, and from undergraduate volunteers: L. Kolody and K. Morden. These collaborators helped by monitoring study subjects and providing support during the complex surgical procedures that I performed for my research.

I was also responsible for analyzing the data contained within Chapter 2. I received advice on choosing the appropriate statistical model, for my desired comparisons, from a few of my colleagues within the lab: Dr. D. Altshuler, Dr. R. Dakin, and D. Skandalis, and an outside member of the faculty: Dr. D. Schluter.

The text within this document are my own original work, with editing provided by Dr. D. Altshuler, Dr. P. Matthews, Dr. R. Shadwick, and Dr. W. Milsom.
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Acknowledgements

I would like to extend my gratitude to the members of my supervisory committee: Dr. D. Altshuler, Dr. R. Shadwick, Dr. W. Milsom, and Dr. P. Matthews for providing me with guidance and wisdom throughout my graduate studies. I would also like to thank my lab mates: M. Armstrong, Dr. J. Bahlman, Dr. V. Baliga, Dr. R. Dakin, J. Enns, Dr. A. Gaede, Dr. B. Goller, C. Harvey, D. Skandalis, G. Smyth, and J. Wong for all their help and support. I would also like to express my appreciation for all the help provided by my two undergraduate volunteers L. Kolody and K. Morden.

I am grateful for the innovation and insight that was provided to me by Dr. D. Syme, my former undergraduate supervisor. Additional thanks to Dr. D. Schluter, whose statistical knowledge was very useful and appreciated.

Special thanks to my parents for their love and understanding throughout my scholarly endeavours.

Finally, a big thank you to NSERC and AFOSR, whose funding allowed me to focus on my research and accomplish my goals.
1 Introduction

1.1 Background and rationale

Birds have been providing inspiration for the design of aircraft for years (Chin et al. 2017). However, their abilities far surpass that of any manmade aircraft, and until we gain a better understanding of the control systems that birds use for flight, they will continue to do so (Chin et al. 2017). One of the areas in which these animals outperform any manmade aircraft is in their ability to achieve high levels of control and maneuverability during flight (Chin et al. 2017). Control of wing shape and size is thought to be the key flight feature that enables birds to navigate through various environments (Altshuler et al. 2015). This aspect of wing control is believed to be attributable to the use of their extensive network of wing muscles throughout the wingbeat cycle (Dial 1992a; Dial 1992b; Robertson & Biewener 2012; Altshuler et al. 2015).

One specific example of wing shape control comes at the level of the elbow joint, since motion in this joint is highly correlated with folding of the wing for the upstroke and re-expansion of the wing for the downstroke. It is believed that there are three muscles that may be responsible for controlling some aspects of active wing shape modulation through regulation of the elbow joint angle: the biceps, and the two heads of the triceps, the scapulotriceps and the humerotriceps. This belief stems from the anatomy of each of these three muscles, which suggests that elbow flexion and wing folding are controlled by the biceps, and that elbow extension and wing expansion are controlled by the two heads of the triceps (Dial 1992a).

Although there is structural and anatomical evidence that suggest that wing configuration is also controlled at the level of the wrist joint (Vazquez 1992; Vazquez 1994; Vazquez 1995), I chose to focus on aspects of control surrounding motion in the elbow joint, primarily for the sake of practicality. There are a lack of measures of the length-change cycle of the wing muscles, relative to the flapping cycle, which provide information that is essential for determining muscle function. These in vivo measures have only
been performed on the wing muscles that surround the elbow joint (Robertson & Biewener 2012), which was what motivated the choice to focus on the elbow joint muscles.

1.2 Muscle properties

During the cyclic contractions that are used for locomotion (Josephson 1985; Josephson 1993; Roberts et al. 1997; Dickinson et al. 2000), a muscle undergoes repeated changes in its length; the muscle is lengthened over one portion of a cycle, and shortened over the remaining portion of that cycle (Josephson 1985). When considering some approximation of steady state locomotion, the ratio of lengthening to shortening can vary, but the result is generally a sinusoidal length-trajectory of constant amplitude (Josephson 1985; Josephson 1993). In cyclic contractions, a muscle contracts and produces force, which can either contribute work to muscle shortening or can provide resistance to lengthening (Josephson 1985; Dickinson et al. 2000; Ahn & Full 2002; Sawicki et al. 2015). The timing of muscle contraction relative to its length-change cycle, and its resultant net work output are dependent on when the muscle is activated during that cycle (Josephson 1985; Ahn & Full 2002; Sawicki et al. 2015).

There is also an inherent delay in muscle force development (Josephson 1985; Full et al. 1998). This means that force development does not occur instantaneously following muscle activation, nor does force production cease immediately after muscle deactivation (Josephson 1993). However, if a cyclically contracting muscle develops force rapidly during rapid lengthening, and this is followed by force being generated primarily over the shortening period, then this would enhance muscle work (Biewener et al. 1998). Stretch and release of a muscle’s series elastic elements during cyclic contractions can also alter force production: stretch during lengthening can slow the contractile element stretch velocity and decrease force, and during shortening the release of energy by elastic recoil can enhance muscle force (Askew & Marsh 1998). Alternatively, muscle stretch and release during a cyclic contraction can contribute to the delay in force development (Josephson 1993; Full et al. 1998). The contractile cycle frequency can
also contribute to force lagging behind the length change of the muscle (Josephson 1993). Therefore, for a muscle to produce maximum positive work during cyclic contractions, it would need to be activated prior to peak length to accommodate the delay in force development (Josephson 1985; Josephson 1993; Full et al. 1998), as this would allow the muscle to produce force solely during the shortening phase of the contractile cycle (Josephson 1993; Tu & Dickinson 1994; Full et al. 1998).

The role of a muscle can be diverse, but again, muscle function is highly dependent on the onset of activation relative to that muscle’s length-change cycle (Josephson 1985; Ahn & Full 2002; Sawicki et al. 2015). A muscle can contribute work to actuate motion, it can absorb work and decelerate motion, or it can provide some other aspect of control by acting as a spring or strut, for example (Josephson 1985; Roberts et al. 1997; Dickinson et al. 2000; Ahn & Full 2002; Sawicki et al. 2015). If a muscle is activated so that it contracts and produces force during the shortening phase of the length-change cycle, then the net work from that cycle is positive and the muscle is serving as an actuator (Josephson 1985; Dickinson et al. 2000; Ahn & Full 2002; Sawicki et al. 2015). Whereas if the muscle is activated such that contraction and higher force production occurs during the lengthening phase, then the resultant net work is negative and the muscle is serving as a brake (Josephson 1985; Dickinson et al. 2000; Ahn & Full 2002; Sawicki et al. 2015). Slight changes to the neural activation duration and timing can therefore cause a muscle to behave differently and cause a shift in the role that it serves (Ahn & Full 2002; Sawicki et al. 2015). Control of muscle activation timing must be precise to move the joint at the desired time (Konow et al. 2015).

The force-velocity relationship in striated muscle indicates an inherent trade-off between force and velocity, such that at a muscle’s maximal shortening velocity force production does not occur, whereas maximal force is produced when shortening velocity is zero (Josephson 1985; Josephson 1993; Askew & Marsh 1998). Given these characteristics of the force-velocity relationship, and that power is the measure of force by distance over time, the resultant power-velocity relationship of any muscle is often parabolic (Josephson 1993; Askew & Marsh 1998). No net power is produced when maximum force is generated,
nor at the maximum shortening velocity, and the optimal velocity that allows maximum power output lies somewhere between these two extrema; near the peak of the curve (Josephson 1993; Askew & Marsh 1998; Ahn & Full 2002). Therefore, changes in the shortening velocity of a muscle during cyclic contractions can alter the power output of that muscle (Josephson 1993; Askew & Marsh 1998; Ahn & Full 2002).

In the avian wing, during flight, the intrinsic muscles are subjected to a length-change cycle that is effectively dictated by the wingbeat cycle (Robertson & Biewener 2012). In addition to having this length change characterized by in vivo measurements, the structure and anatomy of the muscles surrounding the elbow reduce the complexity of measuring work and power output, relative to the muscles surrounding the wrist. Moreover, of the three elbow joint muscles, the humerotriceps muscle is monarticulate, which simplified immobilization of its origin, and allowed for more accurate measures of work and power output. Whereas the scapulotriceps is a bi-articulate muscle, originating on both the scapula and the humerus, which only complicates immobilization of this muscle’s origin. Additionally, isolation and extraction of the muscle insertion was less trivial in the humerotriceps muscle relative to the biceps muscle. Therefore, for anatomically practical reasons, I focused on characterizing the performance of the humerotriceps muscle and how it is used for regulation of the elbow joint angle and control of wing configuration.

In pigeons (Columba livia), the humerotriceps is a bipennate muscle, with a relatively short tendon, that originates on the head of the humerus, and in the pneumatic fossa that is located on the ventral side of the head of the humerus (Robertson & Biewener 2012; Chatterjee 2015). The muscle inserts onto the olecranon process at the proximal end of the ulna on the dorsal side (Robertson & Biewener 2012). The pigeon humerotriceps muscles are activated early during the upstroke through to the mid-downstroke, suggesting that they are actuating and stabilizing elbow extension (Robertson and Biewener 2012). Dial (1992a) also hypothesized that the role of the humerotriceps is to extend the elbow during descending
flight, but that it smooths the transition from upstroke to downstroke, and stabilizes the angle of the elbow during extension and wing expansion (downstroke) during takeoff, landing and ascending flight.

1.3 Study inspiration

The wing muscles that are hypothesized to regulate the elbow joint have been best characterized in the pigeon. The in vivo measurements of the pigeon’s humerotriceps revealed that its activation patterns and length-change cycle, as predicted by the wingbeat cycle, exhibit a large degree of variation (Table 1). In particular, the onset of muscle activation, the duration of that activation, and the frequency of the muscle’s length change cycle showed the most variability both among the different studies and across the different flight modes examined within each of those studies (Dial 1992; Berg & Biewener 2008; Berg & Biewener 2010; Usherwood et al. 2011; Robertson & Biewener 2012). This high level of diversity in the in vivo measurements combined with a muscle’s inherent ability to perform any role suggests that the humerotriceps muscle could be serving different functions for regulation of the elbow joint angle, depending on the flight mode or behaviour. However, the exact nature of the mechanistic control of elbow joint motion in the wing is unknown.

To test these hypotheses of how the humerotriceps is used to regulate the elbow joint angle and provide control of the wing shape, I used some of the variation among these in vivo measures to my advantage. By using them to choose the parameter settings for my work loop study, this allowed me to examine the performance space of the humerotriceps. Currently, there exists no technology that would allow me to a) reproduce the in vivo strain profile and b) simulate the neural activation and motor unit recruitment patterns that occur within the animal when it is using these muscles for flight. While my measures may not allow me to comment directly on the in vivo function of this muscle across different conditions that have been observed during flight, as it would only constitute a weak inference, I can still use the information that I acquired to provide insight into this muscle’s abilities.
Given the structure of the pigeon humerotriceps, its short tendon would allow very little stretch, therefore causing negligible amounts of delay between the onset of force production and initiation of motion (Konow et al. 2015; Sawicki et al. 2015). This made taking measures of force, work and power output from this muscle using the work loop technique ideal since not having to account for tendon stretch made those measures straight-forward. For that reason, I chose to probe these observed areas of variability and examine the performance space of the humerotriceps muscle using the in situ workloop technique (Nelson et al. 2004; Roberts & Azizi 2010). I wanted to use this technique to obtain measures of work and power output that would allow me to determine how these highly variable in vivo measurements of activation onset time, activation duration and contractile cycle frequency in flying pigeons affect the function of the humerotriceps muscle.

1.4 Hypotheses and predictions

1.4.1 Activation onset time

I hypothesized that activation onset timing, or stimulus phase, would shift muscle function by altering the timing of force production relative to the muscle’s contractile cycle, which would result in changes in the net power output of the pigeon humerotriceps. If activation onset timing altered muscle function by shifting force production timing, then I expected that changes to power output would manifest in the following manner: If the muscle is stimulated just prior to peak muscle length (0% phase), then force would be produced primarily during the shortening phase resulting in positive net work and power output. If the muscle is activated after peak length, then the resultant net work and power output will be negative. Positive power output would be consistent with the muscle performing actuation, whereas negative power output would indicate that the muscle is braking (Fig. 1).
Figure 1. Predicted power output from the pigeon humerotriceps muscle in Watts per kilogram (W/kg). Predicted shape of the power output curve for the humerotriceps (black), when measured from in situ work loops across the full range of stimulus onset phases (-50 to 50%). The muscle’s length change above and below resting length (Δ Length; green) in millimeters (mm), is shown for reference. Peak muscle length marks the start of each length-change cycle. Stimulus onset phase is measured as a percentage of that cycle. Positive power output and actuation are expected to occur when the muscle is activated prior to peak muscle length (0% phase), and negative power output and braking would occur when the muscle is activated after peak length. Dashed lines are to show 0 W/kg for power and resting muscle length (horizontal), and peak muscle length and 0% stimulus onset phase (vertical). Minimum muscle length occurs at both -50% and 50% cycle.

1.4.2 Activation duration

I hypothesized that muscle activation duration would alter the magnitude of muscle function in the pigeon humerotriceps by changing the proportion of the contractile cycle over which force production occurs and altering the range of power. If activation duration alters the magnitude of muscle function by changing the range of power output, then reducing stimulus duration would cause an overall increase in the magnitude of the range of power output. The range of power output from a muscle is an important metric because it can provide insight into the range of power produced by the humerotriceps muscle that is potentially available to the animal. I expected the increase in power range to be observable through greater extrema; greater maximum positive and negative power. Since the muscle was being stimulated
for a lower percentage of its length-change cycle, force production would occur over a smaller proportion of that cycle, decreasing the amount of force production overlapping with both the lengthening and shortening phase (Josephson 1993). By reducing this overlap, net positive work and power output will become higher (more positive) and net negative work and power output will become lower (more negative), thus explaining my expectation of an overall increase in the range of the power output curve across the full range of phases (-50 to 50% cycle). This also explains my expectation of an increase in the magnitude of muscle function, because this would mean that more power could be allotted to performing a particular function. Moreover, since the overall range of the power output curve was expected to increase, there would also be an increase in the percentage of stimulus phases over which the humerotriceps produced positive power, and thus a greater range of phases over which actuation could occur. However, during these contractions with constant shortening velocity, shortening-dependent deactivation of the contractile elements could potentially cause force depression and decrease the maximum power output of the muscle (Josephson & Stokes 1989; Josephson 1993; Askew & Marsh 1998; Ahn & Full 2002).

1.4.3 Contractile cycle frequency

I hypothesized that contractile cycle frequency would cause a shift in the function of the pigeon humerotriceps by changing the shortening velocity of the muscle, and altering the range of power of the muscle. If contractile cycle frequency shifts muscle function by changing the muscle’s shortening velocity and altering the power output range, then increasing frequency should cause an increase in the muscle’s range of power output. With stimulus duration held constant at 50% cycle, as frequency increased, the overall range of power output by the humerotriceps muscle would also increase, because of the inherent power-velocity relationship of the muscle (Josephson 1993; Askew & Marsh 1998). I expected that as frequency increased, so would the contraction and relaxation velocities (Josephson 1985), thus causing
an overall increase in power until the optimal shortening velocity of the muscle is reached (Josephson 1993). Increase in shortening velocity beyond the muscle’s optimum, would only cause power output to decrease (Josephson 1993). As a result of the greater range of power, I also expected the percentage of stimulus onset phases where the humerotriceps produced positive power, and served as an actuator, to become greater due to increasing frequency. Again, given the cyclic nature of the muscle contractions, there is still the possibility that positive power output becomes altered by shortening-induced force depression (Josephson 1993; Askew & Marsh 1998; Ahn & Full 2002).
2 Research Chapter

2.1 Introduction

Birds have the ability to achieve high levels of control and maneuverability during flight. This is thought to be attributable to the use of their extensive network of wing muscles that allow them to control the size and shape of their wings throughout the wingbeat cycle (Dial 1992a; Dial 1992b; Robertson & Biewener 2012; Altshuler et al. 2015). One specific example is the motion that is generated at the level of the elbow joint to fold the wing during the upstroke and re-expand it during the downstroke. I focused on wing control endowed by elbow motion because the wing muscles surrounding this joint have been better characterized through \textit{in vivo} measurements than those that surround the wrist joint. Additionally, the structure and anatomy of the elbow muscles makes them more amenable for measures of work and power output. The anatomy of the biceps, and the two heads of the triceps suggests that these three muscles could be responsible for actively controlling the wing shape by regulating the elbow joint angle. However, so long as it is provided with the appropriate activation timing, a muscle can serve any function from actuator to brake, including acting as a spring or a strut (Josephson 1985; Roberts et al. 1997; Dickinson et al. 2000; Ahn & Full 2002; Sawicki et al. 2015). This inherent characteristic of muscles therefore renders the exact mechanism for control of the elbow joint angle unknown.

The wing muscles that are hypothesized to regulate the elbow joint have been best characterized in the pigeon. I focused on characterizing the performance and the potential function of the humerotriceps muscle of the pigeon, due to the availability of \textit{in vivo} measures of both activation and length-change patterns, and for anatomically practical reasons. This muscle’s structure, anatomy, and location made it much easier to obtain measures of work and power output than the biceps or the scapulotriceps. The \textit{in vivo} measurements of the pigeon’s humerotriceps revealed that its activation patterns and length-change cycle, as predicted by the wingbeat cycle, exhibit a large degree of variation (Table 1). In particular, the onset of muscle activation, the duration of that activation, and the muscle’s contractile cycle frequency
showed the most diversity both among the different studies and across the different flight modes examined within each of those studies (Dial 1992a; Berg & Biewener 2008; Berg & Biewener 2010; Usherwood et al. 2011; Robertson & Biewener 2012). The high level of diversity among these in vivo measures suggests that the humerotriceps may serve multiple roles in elbow joint angle regulation, depending on the flight mode or behaviour.

I used the high level of variation among these in vivo measures to choose parameter settings for a work loop study, thereby allowing examination of the performance space of the humerotriceps. Currently, there exists no technology that would allow me to reproduce the in vivo strain profile and simulate the neural activation and motor unit recruitment patterns that occur within the animal when it is using these muscles for flight. Therefore, my measures may not allow me to comment directly on the in vivo function of this muscle across different conditions that have been observed during flight. However, I can still use the information that I acquired to provide insight into this muscle’s abilities. For that reason, I chose to probe these observed areas of variability and examine the performance space of the humerotriceps muscle using the in situ workloop technique (Nelson et al. 2004; Roberts & Azizi 2010) to answer the following three questions:

1. How does stimulus phase affect the power output of the pigeon humerotriceps?
2. How does a reduction in stimulus duration (% cycle) affect muscle power output?
3. How does cycle frequency affect muscle power output when stimulus duration is normalized (% cycle)?

2.2 Methods

2.2.1 Animals

I performed work loop experiments with four female and five male pigeons [Columba livia (Gmelin 1789)] acquired from a local breeder (Aldergrove, BC). The mean body masses were 329.5 grams ± 6.0
sem for females, and 318 grams ± 25.7 sem for males. Birds were housed on site in wire cages, in the Biological Sciences building at the University of British Columbia, with a 12-hour light:dark cycle and ad libitum access to premium pigeon seed mix, water, and two types of grit: mineral and iodine, and calcium enriched grit. All procedures were approved by the University of British Columbia Animal Care Committee (A15-0116).

The work loop study was performed in situ to examine whole muscle performance while maintaining the temperature and the flow of nutrients and oxygen through intact blood supply (Nelson et al. 2004). Birds were anaesthetised prior to the surgery using 4% isoflurane for induction, administered by way of a gas vaporizing system and a 50:50 mix of pure oxygen and nitrogen that was delivered at a combined flow rate of 1L/min. I used 0.6-1.7% isoflurane to maintain the depth of anaesthesia throughout the remainder of the procedure, which was sufficient to keep the animal within the surgical plain. An analgesic, 10%(v/v) butorphanol (1µL/g of bird), was administered intramuscularly via the pectoralis, within an hour of induced anaesthesia. A half-dose of the analgesic was given 4-5 hours after the initial dose. Additionally, 3mL of 0.9% (v/v) saline was administered subcutaneously every 2-3 hours during surgery and just prior to the start of data collection. Any exposed tissue (with skin removed) was covered with 0.9% (v/v) saline soaked cotton. The exposed tissue was also irrigated regularly throughout the surgery with warm saline to clean it and prevent desiccation.

To increase the accuracy of my measures from the humerotriceps, I needed to prevent the surrounding muscles from contributing force, and eliminate any undesired mobility that could create artifacts. The humerotriceps muscle was mostly isolated from surrounding muscles without causing damage to the blood vessels and nerves that supply humerotriceps. The origin of the humerotriceps, which is located on (and in) the head of the humerus, was left intact. The tendon of the humerotriceps is short (~0.5mm) and inserts onto the proximal end of the ulna and was extracted by removing the portion of the ulna where the tendon attaches. I fixed the humerus and immobilized the origin of the
humerotraceps by drilling two small holes in the bone and threading zero gauge 1/4" screws and washers through each hole. These screws affixed the humerus to a 1/4" thick piece of aluminum with pre-tapped holes. The holes in the humerus had approximately 1.5 cm distance between them, with one being located at the distal end of the humerus and the other was proximal to the first, towards the center of the length of the humerus (Fig. 2A).

I activated the muscle via stimulation of the dorsal branch of the brachial nerve, which supplies the humerotraceps (King & McLelland 1984). The nerve was isolated and exposed, by incising the pectoralis major from the wing of interest, and reflecting it away to provide proximal exposure. The brachial nerve was carefully isolated from surrounding connective tissue, tied with 6-0 silk suture, and severed just distal to where it first emerges from the thoracic cavity (Nelson et al. 2004). The nerve was then draped over a bi-polar nerve hook, made from two insulated (HML) silver wires (California Fine Wire) with 1 cm exposed tips that were curved. The two wires were housed in a small syringe with the exposed tips protruding from the tapered end and coated in mineral oil to prevent desiccation without generating a salt bridge between the two electrodes. The electrodes were connected to a High-Power Bi-Phase Current Stimulator (Model 701B, Aurora Scientific Inc., Aurora Ontario). The stimulus pattern was also programmed and input through the protocols written in Dynamic Muscle Control.

2.2.2 In situ muscle work and power measurements

I used the workloop technique (Josephson 1985) to measure work and determine the power output of the whole humerotraceps muscle in situ. This method was an adaptation of the in situ workloop technique used in a previous study of contractile properties of the lateral gastrocnemius and peroneus longus muscles in wild turkeys (Meleagris gallopavo) (Nelson et al. 2004; Roberts & Azizi 2010). For all birds, measures were taken from the humerotraceps muscle in the right wing. The insertion of the humerotraceps was attached to dual-mode lever system arm with a non-compliant, 0.20 mm, thermally
bonded thread (Fig. 2A). The fragment of the ulna was kept intact, and all knots were glued to prevent slippage. The muscle’s temperature was maintained at 36-41°C throughout the experiments.

To record force production and length changes in the humerotriceps muscle I used a Dual-Mode Lever System (Model 305C-LR, Aurora Scientific Inc., Aurora Ontario). This system functions as both a servo motor and a force transducer; in addition to measuring and recording the position and force of the muscle, it receives programmed input to actuate the muscle’s length change. I programmed, ran and monitored all my work loop and parameter optimizing protocols, and treatment sequences using Dynamic Muscle Control (ASI 610A v5.415, Aurora Scientific Inc., Aurora Ontario). Data acquisition was done through an A/D signal interface (Model 604A, Aurora Scientific Inc., Aurora Ontario). All force and length recordings were monitored throughout the experimental procedures using Dynamic Muscle Analysis (ASI 611A v5.200, Aurora Scientific Inc., Aurora Ontario).

I monitored the activation and condition of the muscle using electromyography (EMG) to record the induced muscle potentials of the humerotriceps throughout the duration of the experiments. I made bipolar EMG electrodes from 3-micron diameter HML-insulated silver bifilar wire (California Fine Wires, Grover Beach, CA) with 1 mm tip exposure and 0.5 mm intertip distance (Dial 1992a). This was inserted directly into the humerotriceps. A ground electrode was made from 4-micron diameter insulated silver BI wire (California Fine Wires, Grover Beach, CA) with 1 mm tip exposure, and inserted into the skin, between the left leg and the body. I recorded EMG output using a differential AC amplifier (Model 1700, A-M Systems, Sequim, WA). Live EMG data monitoring was performed during trials using AxoScope (v10.5, Molecular Devices, Inc., Sunnyvale, CA) and a low-noise data acquisition system (Axon Instruments Digidata 1440A, Molecular Devices, Inc., Sunnyvale, CA). The EMG signals were also recorded by the same system that was used to acquire position and force (Aurora Scientific), allowing me to link the muscle activation pattern to the corresponding events in the position and force output traces.
2.2.3 Work loop Parameters

I performed a set of parametric tests to optimize the performance of the humerotriceps muscle prior to collecting data. I determined the appropriate stimulus intensity that would generate maximum force production through a series of two-pulse isometric contractions. During these contractions, voltage was held constant and current was increased (Lacourpaille et al. 2013). I increased the current by increments of 1mA until maximal force output was achieved. I then increased the current by another 50%, and used this intensity for the remainder of the experiment (Josephson 1997). This procedure should ensure full motor unit recruitment, while maintaining longevity of the muscle and nerve. I ran two tetanic contractions and one to two work loops to get an estimate of the maximum force production of the muscle, and to check that the muscle, the knot, the humerus were all secure and properly immobilized before starting the next phase of measurements to evaluate the force-length relationship of the muscle.

I evaluated the force-length relationship of the humerotriceps muscle using a series of two-pulse isometric contractions. Between measurements, I increased the length of the muscle by shifting the motor arm away from it in 0.5-1 mm steps. I calculated the difference between the passive tension (baseline) and peak force both before and after the contraction (Josephson 1997), which is also known as the developed force. The motor arm position and muscle length were then chosen based on a balance between maximized force production and low passive tension. This balance was determined when the developed force no longer exhibited an increase with increasing length. If there was no apparent developed force plateau, the muscle was lengthened until the increase in developed force was negligible relative to the previous length (i.e.: ~50 mN) due to a near parallel rise in passive tension. In these cases, the shorter of the two muscle lengths exhibiting similar developed forces was chosen.

I used one standardized work loop protocol as a control (baseline) to monitor the condition of the muscle and degradation of force across the duration of each experiment. The settings for the control work loop protocol were 8.6 Hz length-change cycle frequency, stimulus duration covering 69% of the length-
change cycle, 8.7% strain amplitude, and stimulus onset at -30% phase. The control work loop settings were chosen based on *in vivo* measures of muscle length-change cycle frequency, stimulus duration and strain amplitude (Robertson & Biewener 2012). I chose the stimulus onset phase based on preliminary data that showed net positive work and power output consistently reached the highest values at -30% phase. Strain amplitude refers to the overall percent of muscle length change, or the difference between maximum and minimum length (Robertson & Biewener 2012). Here I use the definition of activation, or stimulus onset phase provided by Robertson & Biewener (2012), where it is the percentage of the length-change cycle at which stimulus onset occurs, and 0% cycle is defined as peak muscle length (Fig. 2B).

I experimentally manipulated some work loop parameters, chosen based on previous literature that suggested the range of *in vivo* conditions under which the pigeon humerotriceps muscle is operating (Table 1). The measures that exhibit the most variation are the activation onset as a percentage of the muscle length-change cycle, activation duration as a percentage of the wingbeat cycle, and wingbeat cycle frequency (equivalent to the wing muscle length-change cycle frequencies; Robertson & Biewener 2012). I chose my work loop treatment parameters using ranges that would reflect these observed variations, leading to four different treatments (Table 2). The two studies that most strongly influenced work loop variables were Dial (1992a) and Robertson & Biewener’s (2012) (Table 1).

I examined how muscle performance was affected by stimulus timing by testing across the full range of stimulus phases, from -50% to 50% of the muscle length-change cycle at 5% intervals, for a total of 21 different stimulus onset phases (Table 2). One adjustment was that 10% phase was replaced by 9% to represent the average *in vivo* activation onset reported by Robertson & Biewener (2012). Although the timing of stimulus onset relative to peak length is the same for -50 and 50% phase, their work loop protocols differ slightly. Work loop trials set at -50% phase receive stimulation right at the outset of the muscle length-change cycle, whereas stimulation is delayed by one full length-change cycle for 50% phase. All experiments presented in this study included measurements at each of the 21 stimulus phases. Within
each set of measurements, stimulus onset phases were tested in randomized order, and the same random order was held constant for each experimental treatment. Every experimental treatment started and ended with -30% phase as an additional check for force degradation, for a total of 22 work loop trials per individual per treatment.

I examined how stimulus duration affected muscle performance by varying normalized stimulus duration. The normalized stimulus duration is determined as the percentage of the length-change cycle over which the muscle is being stimulated, meaning that the absolute stimulus duration in milliseconds (ms) varies with cycle frequency (Fig. 2C). I decided to consider normalized rather than absolute stimulus durations because in vivo observations from both Dial (1992a) and Robertson and Biewener (2012) indicate that in general, stimulus duration varies as a function of cycle frequency. I tested two normalized stimulus durations, 50 and 69% cycle (Table 2). 50% duration matched in vivo EMG results from Dial (1992a), whereas 69% matched in vivo results from Robertson and Biewener (2012) (Table 1).

I examined how wingbeat frequency affects muscle performance by varying work loop cycle frequency while holding stimulus duration constant at 50% of the cycle (Table 2). My main muscle length-change cycle frequency was chosen based on the average frequency (8.6 Hz) reported by Robertson & Biewener (2012). I looked at two other frequencies, 6.1 and 10.1 Hz, which represent the minimum and maximum wingbeat frequencies recorded from pigeons across several studies (Table 1).

Some work loop parameters, specifically strain amplitude, stimulus pulse duration and pulse frequency, and number of strain cycles per trial, were held constant across all treatments, including the control. Strain amplitude was held constant because, due to the location of the origin of the humerotraceps, muscle length could not be accurately measured until after the experiments were completed. Therefore, I determined the strain amplitude to be used for all work loops by calculating 8.7% of the average resting length of the humerotraceps muscles from my previous experiments. I used a set stimulus pulse duration of 0.2 ms and pulse frequency at 300 Hz (Bahlman & Altshuler, in review). The
number of pulses per train varied depending on the stimulus duration (% cycle), and depended on the length-change cycle frequency. Every work loop trial, including the control, contained either five or six work loops per trial, regardless of the treatment. This was due to stimulus timing relative to the muscle length-change cycle. Although all work loop trials contained six length-change cycles, for work loop trials where stimulus phase occurred prior peak length (negative phases) the muscle was stimulated during all six cycles, and therefore these trials contained six work loops. Additionally, due to timing, muscle stimulation was incomplete during the first length-change cycle of most of these negative stimulus phases. Work loop trials where stimulus phase occurred after peak length (positive phases) only had five length-change cycles where the muscle was stimulated, and therefore only contained five work loops.

Currently, there is no way to program complex strain profile waveforms that would better approximate the *in vivo* strain profile of the pigeon humerotriceps muscle. Moreover, inspection of the raw sonomicrometry data that were measured *in vivo* from the pigeon humerotriceps (Robertson & Biewener 2012), revealed that there exists some level of individual variation with respect to the shape of this muscle’s strain profile. Although the trajectory of the two *in vivo* strain profiles differed, they still retained a sinusoidal shape. Therefore, during my work loop experiments the strain profile was approximated by a sine wave with a 50:50 lengthening to shortening phase ratio. I used this as the strain profile for all work loop trials since the *in vivo* muscle strain profile had an approximate lengthening to shortening phase ratio of 45:55 (Robertson & Biewener 2012), which is not far off from the 50:50 ratio that I chose.

Prior to analysis, I performed several validity checks of the humerotriceps work loop data using custom scripts written in MatLab (MatLab R2016b, MathWorks Inc., Natick, MA). First, I examined the EMG, length and force traces of each individual work loop trial to check for anomalies that could result from knot slippage, muscle tearing, or loss action potential propagation in the muscle. I next visually inspected work loops three through five in each trial, to ensure that none of the work loops from an
individual, within a given phase treatment differed substantially from one another. In terms of their shape and magnitude, work loops three through five of a given treatment should not vary much among individuals either. Finally, I examined the peak forces produced during the control work loops, which were run before and after each treatment. The net work and power output of the control work loops were not always positive. Therefore, I compared the means of peak forces of each control work loop to determine whether a given treatment should be included in the final dataset. If the peak forces of a control work loop were ≥59% of the initial control then the treatment that was run just prior to that control was considered valid. If a control work loop had a mean peak force <59% of the initial control, the treatment that directly preceded it was deemed unreliable and was omitted from the final dataset.

I summarized the remaining data to improve the accuracy of my measures of the humerotriceps power output across the different treatments. I averaged the power outputs across work loops three through five within each trial for a given stimulus onset phase for consistency and because not all trials contained six work loops. These three values were also consistent and included the maximum values within each of the first set of five work loops (Fig. 3).
Figure 2. Methods for examining the effects of contractile frequency and stimulus duration on the power output of the pigeon humerotriceps muscle. 

A. The humerotriceps muscle originates on the head of the humerus and, during the in situ work loop experiments, the insertion is attached to the servo motor arm. Circles show where screws were drilled into the humerus to immobilize it. 

B. A sample length-change cycle trace plotted over time, with gray dashed lines indicating where stimulus onset began for -50, -25, 0 (peak muscle length) and 50% phase. 

C. Length-change traces of the three different frequency treatments: 6.1 Hz (top, black); 8.6 Hz (middle, red) and 10.1 Hz (bottom, blue). The stimulus duration bars below each of the traces (A-C) shows that although the cycle frequency-normalized stimulus duration is held constant, when measured in milliseconds, stimulus duration varies as a function of cycle frequency. Contractile frequency was tested with 50% stimulus duration. The effect of stimulus duration (50% versus 69%) was tested at a cycle frequency of 8.6 Hz.
Figure 3. Work loop results were consistent across individuals. Work loops three through five from all trials in the study are plotted as force (N) by length (Δlength mm) traces. The x-axis indicates stimulus onset phases (-50 to 50%) across all four experimental treatments. Nine individuals were tested, but only two were tested for two treatments, and the n value for each treatment ranged from two to four. A. Work loop traces at 8.6 Hz and 69% stimulus duration. B. Work loop traces at 6.1 Hz and 50% stimulus duration. C. Work loop traces at 8.6 Hz and 50% stimulus duration. D. Work loop traces at 10.1 Hz and 50% stimulus duration. Scale bars for both force and length are plotted with the leftmost work loop (-50% phase) of every work loop series and hold for that entire row.
Table 1. Flight and muscle variables. Flight and muscle variables measured in free-flying* and captive, trained pigeons**.

<table>
<thead>
<tr>
<th>Study</th>
<th>Flight Mode</th>
<th>Wingbeat</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Frequency (Hz)</td>
<td>Duration (ms)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Robertson &amp; Biewener 2012**</td>
<td>Takeoff</td>
<td>8.6</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>Level flight</td>
<td>(averaged across flight modes)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Landing</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>Usherwood et al. 2011*</td>
<td>N/A</td>
<td>5 to 10</td>
<td>200 - 100</td>
</tr>
<tr>
<td>Berg &amp; Biewener 2010**</td>
<td>Takeoff</td>
<td>7.1 to 9.2</td>
<td>113 - 130</td>
</tr>
<tr>
<td></td>
<td>Level flight</td>
<td>6.57±0.30</td>
<td>152±3.33</td>
</tr>
<tr>
<td></td>
<td>Landing</td>
<td>6.1 to 8.0</td>
<td>128 - 147</td>
</tr>
<tr>
<td>Berg &amp; Biewener 2008**</td>
<td>Ascent and Descent</td>
<td>6.1 to 9.6</td>
<td>104 - 164</td>
</tr>
<tr>
<td>Biewener et al. 1998**</td>
<td>Level flight</td>
<td>8.70±0.26</td>
<td>115±3.85</td>
</tr>
<tr>
<td>Dial 1992a**†</td>
<td>Takeoff</td>
<td>9.1 (range: 8.3 to 10)</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Ascent</td>
<td>9.4 (range: 9 to 10.1)</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>Level flight</td>
<td>8.5 (range: 7.6 to 9.3)</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>Descent</td>
<td>8.4 (range: 7.7 to 9.5)</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>Landing</td>
<td>8.3 (range: 7.5 to 9.3)</td>
<td>120</td>
</tr>
</tbody>
</table>

† All values were estimated using the published figures, and then used to calculate activation duration as a percent of the wingbeat cycle, and average wingbeat frequency and duration (Fig. 8 and 10, respectively, from Dial 1992a).

‡ Ranges calculated using average humerotriceps peak length (% relative to the wingbeat cycle) reported by Robertson & Biewener (2012) and the onset timings from the respective studies.

†† Ranges determined using the humerotriceps peak length (% relative to the wingbeat cycle) calculated from raw sonomicrometry data (Robertson and Biewener 2012), across each flight mode.

††† Dial (1992a) defined the start of each wingbeat cycle as the onset of pectoralis activation, whereas Robertson & Biewener (2012) defined it as the upstroke-downstroke transition. To justify using the humerotriceps peak length (% wingbeat cycle) from the latter study to calculate Dial’s (1992a) activation onset timings (% muscle length-change cycle), the start of the wingbeat cycle was shifted by 21% to reflect start of the downstroke (Dial 1992a).
Table 2. Experimental treatments and work loop parameters. This study contained four different experimental treatments to answer three questions: 1. *How does stimulus phase affect the power output of the pigeon humerotriceps?* 2. *How does a reduction in stimulus duration (% cycle) affect muscle power output?* 3. *How does cycle frequency affect muscle power output when stimulus duration is normalized (% cycle)?* Treatment indicates the work loop parameters that were varied. The research questions that they addressed (Questions), the subjects that they were measured from (Birds) and the total sample size per treatment (n) are also outlined. Stimulus onset phases were examined at the same intervals for all experimental treatments.

<table>
<thead>
<tr>
<th>Question(s)</th>
<th>Treatment</th>
<th>Cycle Frequency (Hz)</th>
<th>Normalized stimulus duration (% cycle)</th>
<th>Stimulus onset phases (% cycle)</th>
<th>Birds</th>
<th>Sample size per treatment (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 2</td>
<td>Stimulus phase and duration</td>
<td>8.6</td>
<td>69</td>
<td>-50 to 5, 9, 15 to 50</td>
<td>1-4</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Stimulus phase and cycle frequency</td>
<td>6.1</td>
<td>50</td>
<td>-50 to 5, 9, 15 to 50</td>
<td>1,4</td>
<td>2</td>
</tr>
<tr>
<td>2 and 3</td>
<td>Stimulus phase, duration and cycle frequency</td>
<td>8.6</td>
<td>50</td>
<td>-50 to 5, 9, 15 to 50</td>
<td>5,6</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Stimulus phase and cycle frequency</td>
<td>10.1</td>
<td>50</td>
<td>-50 to 5, 9, 15 to 50</td>
<td>7-9</td>
<td>3</td>
</tr>
</tbody>
</table>

2.2.4 Statistical analysis

To address my first question of how stimulus phase affects the power output of the pigeon humerotriceps, I analysed the power output of the humerotriceps across 21 stimulus phases by fitting these data with a linear mixed effect model (LMEM). I then compared this model to a null model that excluded phase as an explanatory variable, using likelihood ratio test. This method allowed me to compare power output among the different stimulus phases within each treatment (Table 2), while taking into account that there were repeated measures and autocorrelation over the duration of an experiment.
I addressed my second and third questions of how reduced stimulus duration (% cycle) affects the muscle power output and of how cycle frequency affects the muscle power output by fitting the data from each of these two treatments with a Generalized Additive Model (GAM). The GAMs were fit for the power output across the 21 different stimulus phases. These models provided visual and quantitative characterization of the changes in power output that occur across the different treatment groups. I used the individual fits of each model to derive four summary statistics that quantify changes in shape of the power output curve: 1) mean power over all 21 phases, 2) maximum and 3) minimum power output, and 4) the percentage of the full stimulus phase cycle over which positive power is produced, hereafter referred to as actuation percentage. These measurements illustrate the effects of varying normalized stimulus duration and cycle frequency on power output across all 21 stimulus phases. It is important to note that the mean power output at -30% phase was determined from both the first and the last trials within a treatment because this stimulus phase protocol was used to check for degradation every work loop treatment. I used an analysis of variance (ANOVA) to compare each of the four summary statistics within each of the two treatments. All statistical analyses were performed using R (version 3.3.2, R Development Core Team, 2016).

2.3 Results

The work loop technique provides measurements of how muscle force changes during the shortening-lengthening cycle, and further allows for other muscle performance measurements across the full cycle including maximum and minimum force, work, and power output. Representative results obtained through the in situ work loop technique are provided in figure 4. The time-dependent changes in force (N) and muscle length (mm) are given for six consecutive length-change cycles of a single trial (Fig. 4A). Due to differences in the number of cycles where the muscle received complete stimulation, which resulted from varying stimulus timing, the first five cycles of each trial were used for consistency. Plotting
this data sequence as force versus length produces five work loops (Fig. 4B). The area within each work loop is integrated to determine the net work output of each muscle contraction cycle (Fig. 4C). Absolute net work increases with cycle number but approaches an asymptote for the last three work loops within a trial (Fig. 4C). These three cycles were therefore used to calculate the mean power output of the humerotriceps muscle during each trial.

The experiments were designed to examine the role of stimulus onset phase, stimulus duration, and cycle frequency.
Figure 4. Representative work loop recording of the pigeon humerotriceps muscle during one control trial. A. The raw force in Newtons (N, gray) and length in millimetres (mm, black) are plotted as a function of time. The thick bands on the length trace indicate the period when stimulus was applied to the nerve to activate the muscle. B. The resultant work loop traces are plotted as force against the change in length. The arrows indicate the direction of the loops, and counter-clockwise loop directionality indicates that the net work in millijoules (mJ) is positive. The first loop is shown in red because muscle stimulation was incomplete and therefore it does not follow the same trajectory as the other work loops and it does not close. C. The net work (mJ) output from each of the five work loops within one trial is determined from the integrated area within each loop. The net work from the first loop was negative and is shown in red. The circle with the dashed line indicates the three work loops within a trial that are used to calculate mean power in Watts per kilogram (W/kg) for that trial. The control work loop trials were run in between experimental treatment sequences to check the force degradation. All control work loops were run at a cycle frequency of 8.6 Hz, a stimulus onset phase of -30% cycle, and a stimulus duration of 69% cycle.
2.3.1 Stimulus onset phase

I used the work loop technique to examine the full range of stimulus onset phases to cover the high level of variability in the in vivo measurements of activation onset timing in flying pigeons. The work loop technique provides measures of the changes in muscle power output with shifts in neural activation timing. Stimulus onset phase had a significant effect on the power output of the humerotriceps muscle ($P < 0.0001$). Comparing power output across the full range of stimulus onset phases led to a response curve (Figs. 5 & 6A) that was similar in shape to that which I had predicted (Fig. 1).

The greatest positive power output was produced by the humerotriceps muscle when stimulated between -40 and -35% cycle (24.6 ± 15.5 W/kg and 30.1 ± 15.5 W/kg, respectively). Power output was most negative between 5 to 15% phase (-190 ± 15.5 W/kg and -164 ± 16.7 W/kg, respectively). The response curve of the humerotriceps was characterized by its production of positive power only when stimulated prior to peak length (0%), and as the phase approached and surpassed 0%, power became increasingly negative, until it reached the minima, after which it began to increase again and shift back towards zero (Fig. 5). Slight deviations from this trend occurred at 40 and 50% stimulus onset phase, where power output was notably lower than that of the neighbouring phases, but not lower than the minima (Fig. 5).
Figure 5. Power output in Watts per kilogram (W/kg) from the pigeon humerotriceps muscle at each of 21 different stimulus onset phases (% cycle). Symbols indicate power output data from four different individuals. The gray shaded area indicates the phases at which mean power output (± sem) is positive, i.e., above zero (n=4 for all phases except -45, 0, 15 and 40%; n = 3). The effect of stimulus onset phase (from -50 to 50% cycle) was tested using a cycle frequency of 8.6 Hz, and a stimulus duration of 69% cycle.

2.3.2 Stimulus duration

I examined two different values for stimulus duration (50% and 69% of cycle duration), and tested each across the full range of stimulus phases. Muscle power output was significantly affected by stimulus duration (P < 0.0001 for both stimulus durations) but both power output curves were similar in shape across the full range of stimulus onset phases. Moreover, the peak, trough, and crossover regions of the power output curve were similar. As with the power output across all phases at a stimulus duration of 69% cycle (Fig. 6A), at 50% cycle, the power output of the humerotriceps was only positive when stimulus phase was highly negative (Fig. 6B). Power output was highest around -40% phase, and lowest at around 5% phase (Fig. 6B & D).
The primary effect of reducing stimulus duration from 69% to 50% of the cycle was to increase the range of the humerotriceps power output curve (Fig. 6). The maximum power output represents the highest level of actuation, whereas minimum power output represents the highest level of braking, and the lower stimulus duration had the higher extrema (Fig. 6D). However, although there was a decrease in maximum power as stimulus duration was increased from 50% to 69% cycle (Fig. 6D), the difference between the two maxima was marginally insignificant ($P=0.059$). The minimum power output did however, show a significant increase when stimulus duration was shifted from 50% to 69% cycle ($P=0.0048$). The minimum power output at 50% stimulus duration was $-300.6 \pm 19.0$ W/kg, which was nearly double the minimum power output of $-183.5 \pm 11.4$ W/kg, at 69% stimulus duration (Fig. 6D).

Comparison of the mean power, and the actuation percentage allows for quantification of any overall shift in the power output curve between the two treatments (Figs. 6C & E, respectively). The mean power output of the two treatments did not differ significantly across all 21 stimulus onset phases ($P=0.395$; Fig. 6C). The actuation percentage also did not differ among the two treatments (Fig. 6E; $P=0.219$).
Figure 6. Reducing the stimulus duration of the pigeon humerotriceps muscle increased the range of power output values across the different stimulus onset phases. Power output of the humerotriceps muscle across the full stimulus phase cycle (-50 to 50%) was tested at two normalized stimulus durations of 69% cycle (A, purple, n=4) and 50% cycle (B, red, n=2). Symbols represent power output data from different individuals, and the curved lines show the fitted generalized additive model (A and B). The three panels on the right (C-E) are the summary statistics determined from the fitted model. C. The mean power output (W/kg ± sem) across all 21 phases (-50 to 50%) within a treatment sequence. D. The range of power (W/kg ± sem), where maximum actuation is the maximum positive power produced by the humerotriceps, and maximum braking is the minimum (most negative) power. E. The percentage of stimulus onset phases (± sem) over which the net power output is positive. The effect of stimulus duration was tested at a cycle frequency of 8.6 Hz, using 21 different stimulus onset phases that spanned from -50% to 50% cycle.
2.3.3 Frequency

I tested three values of cycle frequency that represent the minimum, mean, and maximum values determined from in vivo measurements of flying pigeons. Cycle frequency affected humerotriceps power production by increasing the overall range of the power output curve with increasing frequency (Fig. 7). Despite this increased range, power produced by the humerotriceps was still increasingly negative as frequency increased due to an overall downward shift in the power output curve (Fig. 7).

The power output by phase curves retained overall similarities in their shape, despite changes to the cycle frequency. This included the retention in location of the peak, trough and crossover regions. Varying stimulus onset phase had a significant effect on the power output of the humerotriceps within each frequency treatment (P = < 0.0001 for all three frequencies). Positive power was only produced when stimulus onset was much before 0% phase, and as onset timing approached and surpassed 0%, power output decreased. Maximum negative power output occurred between 5 and 20% phase, after which it would start to climb toward zero again. Some differences among the power output curves of these three frequency treatments included slight shifts in the phases at which the maxima and minima occurred. At 6.1 Hz, the greatest power output was produced at around -40% phase, and the lowest power output occurred at 20% phase. At 8.6 Hz, the highest power output was also around -40% phase, and the lowest was around 5% phase. Finally, at 10.1 Hz, the highest power output was observed at around -30% phase, and as was the case for 8.6 Hz, the lowest power output occurred around 5% phase.

Changes in the overall range of the power output curve among the three frequency treatments were again quantified through comparisons of maxima and minima. As cycle frequency was increased from 6.1 Hz to 10.1 Hz, there was an increase in the maximum power output from 49.4 ± 4.44 W/kg to 93.2 ± 13.4 W/kg (Fig. 7E), but this difference was marginally insignificant (P = 0.055). Increasing cycle frequency caused a significant decrease in minimum power (P = 4.77e-05). The negative power output was smallest at 6.1 Hz (-96.0 ± 4.44 W/kg), and at 8.6 Hz the negative power output more than tripled to -301 ± 19.0
W/kg and finally, at 10.1 Hz, the humerotriceps produced -510 ± 13.4 W/kg which was more than five times greater than at 6.1 Hz (Fig. 7).

Shifts in the power output curve across the three frequency treatments were quantified using the actuation percentage and the mean power across the full suite of stimulus onset phases. Across the three frequency treatments, the actuation percentages showed a slight decrease with increasing frequency (Fig. 7D), but these differences were marginally insignificant (P = 0.055). The mean power output exhibited significant changes among the three frequencies (P = 0.00044). Mean power output was greatest at 6.1 Hz (-20.4 ± 4.44 W/kg), and declined to -174 ± 13.4 W/kg at 10.1 Hz (Fig. 7F).
Figure 7. Power output from the pigeon humerotriceps decreased with increasing cycle frequency. Power output of the humerotriceps muscle across the full stimulus phase cycle (-50 to 50%) was tested at cycle frequencies of 6.1 Hz (A, black; n=3), 8.6 Hz (B, red; n=2), 10.1 Hz (C, blue; n=3). Symbols represent the power output data from different individuals, and the curves indicate the fitted generalized additive model (A-C). The three panels on the right (D-F) are the summary statistics determined from the fitted model. D. The mean power output (W/kg ± sem) across all 21 phases (-50 to 50%) within a treatment sequence. E. The range of power (W/kg ± sem), where maximum actuation is the maximum positive power produced by the humerotriceps, and maximum braking is the minimum (most negative) power. F. The percentage of stimulus onset phases (± sem) over which the net power output is positive. Contractile frequency was tested at a stimulus duration of 50% cycle.
2.4 Discussion

The pigeon humerotriceps is a bipennate muscle with a relatively short tendon. It originates on (and in) the head of the humerus, and inserts onto the olecranon process, at the proximal end of the ulna (Robertson & Biewener 2012). The location, structure and inherent properties of this muscle suggest that it plays a role in elbow joint motion and stability (Dial 1992a). To gain a better understanding of the role of the humerotriceps muscle, I used knowledge of the in vivo conditions under which it operates to measure muscle force dynamics using the in situ work loop technique (Nelson et al. 2004; Roberts & Azizi 2010). This method was suitable given that the tendon of the humerotriceps is short and would produce very little stretch and therefore cause a negligible amount of delay between force production and motion (Konow et al. 2015; Sawicki et al. 2015).

2.4.1 Stimulus onset phase

The first question I addressed was how does stimulus phase affect the power output of the pigeon humerotriceps? My hypothesis was that varying the stimulus phase would shift the function of the pigeon humerotriceps by altering the muscle’s force production timing and the power output. I predicted that if the muscle function was affect by stimulus phase, then when the muscle was stimulated prior to peak muscle length (0% phase), force production would occur during muscle shortening and result in net positive power output. Alternatively, if the muscle was activated after peak length, then the resultant net power output would be negative. Positive power output would suggest that the muscle serves as an actuator, whereas negative power output would suggest that the muscle serves as a brake (Fig. 1). To test my hypothesis and predictions, I used the in situ work loop technique, with a contractile cycle frequency of 8.6 Hz and stimulus duration of 69% cycle and measured the force and power output of the humerotriceps muscle at stimulus phases ranging from -50 to 50%.

I found that varying phase had a significant effect on the power output of the pigeon humerotriceps muscle, and the resultant power output across the full range of phases (Fig. 5 & 6A) exhibited a curve that
was similar to what I had predicted (Fig. 1). When a muscle is activated just prior to its peak length it can produce net positive power, and behave as an actuator (Josephson 1985; Josephson 1993; Dickinson et al. 2000; Ahn & Full 2002; Sawicki et al. 2015). However, if activation onset occurs too early or too late relative to peak muscle length, this causes force production to shift and overlap the lengthening phase. Continuing to shift activation onset, such that greater overlap of force production with the lengthening phase occurs, decreases power output until it eventually becomes net negative, indicating that the muscle is absorbing work and serving as a brake (Josephson 1985; Josephson 1993; Dickinson et al. 2000; Ahn & Full 2002; Sawicki et al. 2015). This was exactly what I observed from varying phase during my work loop experiments on the pigeon humerotriceps.

However, it should be noted that the range of phases over which positive power was produced by this muscle was much narrower than the range of phases over which negative power output occurred. This was likely primarily due to the relatively long stimulus durations. However, this may also have resulted from the use of a sine wave strain profile with a 50:50 lengthening to shortening ratio, when in vivo the humerotriceps spends slightly less time in the lengthening phase and more time in the shortening phase (Robertson & Biewener 2012). The reduced shortening phase may have inadvertently reduced the actuation percentage of the humerotriceps. For the muscle to produce positive power, force generation must occur during muscle shortening (Josephson 1985; Josephson 1993; Sawicki et al. 2015). By assuming that the lengthening and shortening phases were equivalent, I reduced the shortening phase relative to what was found in vivo (Robertson & Biewener 2012). In doing so, I had placed limitations on the muscle’s ability to produce positive power (Josephson 1993; Askew & Marsh 1998; Ellerby & Askew 2007; Sawicki et al. 2015).

An additional caveat of the in situ technique is that the differences in power output at -50 and 50% phase may potentially be attributed to slight differences in the way that each of the two work loop trials were run (Fig. 5). Since at -50% phase, stimulus was initiated earlier in the trial than trials run at 50%
phase, then power output differences could have been generated from calculating mean power from the cycles three through five for each trial. At -50% phase, using cycles three through five meant that the last three cycles within this work loop trial were used to determine mean power output, and likely would have contained the maximum work and power output value for this phase. Conversely, at 50% phase cycles four through six would have been the last three cycles, therefore using cycles three through five may have inadvertently omitted the maximum work and power output value for this phase. However, these differences also could have been an artifact of the order in which the work loop trials were run. Although the two phases were close in sequence to one another; -50% was eighth and 50% was twelfth in each treatment sequence, this may still have contributed to the differences in power output. Force degradation was time dependent during these experiments, therefore despite their proximity in the sequence, this subtle difference may have been a causal factor. Stretch enhancement of force during lengthening could have increased the negative work and power output more prominently at 50% stimulus phase. Alternatively, stretch enhancement of force during shortening could have reduced negative work and power output more prominently at -50% phase. The reason for the difference in power output between these two phases is unclear.

2.4.2 Stimulus duration

The second question that I addressed was how does a reduction in stimulus duration (% cycle) affect muscle power output? My hypothesis was that variation in stimulus duration (% cycle) would change the magnitude of function in the pigeon humerotriceps by altering the proportion of force production relative to the length-change cycle and resulting in a shift in the range of power output of the muscle. I predicted that if stimulus duration affected the magnitude of muscle function, then reducing stimulus duration would decrease the duration of force production, resulting in fewer stimulus phases where force production overlapped with both the shortening and the lengthening phase of the muscle’s contractile
cycle (Josephson 1993; Askew & Marsh 1998). Thus, for any given stimulus phase, the net work and power output should exhibit an overall increase, thereby increasing the magnitude of muscle function when a shorter stimulus duration was applied.

To address my second question, I tested the results from my first question against the same work loop treatments run at 50% cycle stimulus duration. Testing the effects of reduced stimulus duration was again done using the *in situ* work loop technique on the pigeon humerotriceps. I found that at a reduced stimulus duration of 50% cycle, the power output range exhibited an increase relative to that observed at 69% cycle stimulus duration (Fig. 6) as predicted. The increase in power range was likely a result of the shorter (50%) stimulus duration causing force production over a shorter period of the length-change cycle (Josephson 1993). Although the range did exhibit an overall increase, this increase was due primarily to the differences in minimum power output among the two stimulus duration treatments. The negative power output was much greater at the 50% cycle stimulus duration than at 69% cycle (Fig. 6D). The lack of significant increase in the maximum power output with decreased stimulus duration (Fig. 6D), and the absence of any changes to the actuation percentage (Fig. 6E) may be attributable to the strain profile that was used for these work loop treatments. Again, the chosen strain profile assumed that lengthening and shortening phases are equivalent and that the strain trajectory is a sine wave. Positive power output may also have remained unchanged as a result of force depression caused by shortening-dependent deactivation of the muscle’s contractile elements (Josephson 1993; Askew & Marsh 1998; Ahn & Full 2002). Because of the cyclic nature of the work loop contractions that the humerotriceps was subjected to, shortening-induced deactivation may have limited the force and ultimately the positive power output of this muscle (Josephson 1993; Askew & Marsh 1998; Ahn & Full 2002).

Slight deviations from the *in vivo* strain profile may have been enough to limit the positive power output of this muscle (Josephson 1985; Josephson 1993). This explains why only a small range of stimulus
phases exhibit positive net power output, and why maximum power did not differ between the two stimulus duration treatments (Fig. 6E & D, respectively). Conversely, reduction of the shortening phase relative to in vivo means that force production can occur over a greater portion of the lengthening phase, which would result in increased negative power output (Josephson 1993; Askew & Marsh 1998).

There were again, differences between the power output at -50% stimulus phase and 50% phase. However, with a stimulus duration of 50% cycle, the power output at -50% was positive, whereas at 50% phase it remained highly negative (Fig. 6B). Again, this difference in power output may have been due to the differences between the two work loop trials, an artifact of the trial sequence within each treatment, or perhaps caused by differential stretch enhancement of force. The shorter stimulus duration (50% cycle) may be what allowed positive power output at -50%; force production was shorter and thus less likely to overlap with the lengthening phase (Josephson 1993), and stimulus onset occurred early in the contractile cycle, prior to peak length (Fig. 6B). Additionally, if differences in stretch enhancement were the source of the discrepancies in power output of these two phases, then decreasing negative work at one phase (-50%) but not the other, could explain why -50% phase exhibited positive power output while 50% phase did not (Fig. 6A & B).

2.4.3 Frequency

The third and final question I addressed was how does cycle frequency affect muscle power output when stimulus duration is normalized (% cycle)? My hypothesis was that cycle frequency would alter the function of the pigeon humerotriceps by changing the shortening velocity and the range of power output of the muscle. I predicted that if contractile cycle frequency affected muscle function in this way, then as frequency increased the muscle would approach its optimal shortening velocity and its maximum power output (Josephson 1993), thus increasing the magnitude of actuation. I also predicted that increased frequency would cause a higher range of power, which would result in an increase in the actuation
percentage. As predicted, I did see an increase in the range of power output as frequency was increased from 6.1 to 10.1 Hz (Fig. 7A-C & E). However, the actuation percentage did not significantly increase with increasing frequency (Fig. 7F). Lastly, there was a significant decrease in the mean overall power output that occurred as frequency increased (Fig. 7D).

It should be noted that the increased range of power with increasing frequency was significant because the minimum power output became increasingly negative (Fig. 7E). Although increased frequency caused the overall power output to increase, the maximum power output did not change (Fig. 7E). This was potentially a by-product of the strain profile, where the shortening phase was already slightly less than what has been observed in vivo (Robertson & Biewener 2012). Increasing the frequency further reduced the duration of the shortening phase, and force production overlapped the lengthening phase within a greater number of the stimulus onset phases (Josephson 1993; Askew & Marsh 1998). Additionally, depending on the force-velocity relationship of the humerotriceps muscle, increasing the shortening velocity can also cause work and power output to decrease (Josephson 1993; Askew & Marsh 1998; Ahn & Full 2002). For phases that produced net positive power, this effectively reduced their power output (Fig. 7A-C & E). As a result, there was no difference in the maximum power output at the different frequencies (Fig. 7E).

The absence of significant change in the actuation percentage, and the significant increase in negativity of the mean power with increased frequency did not match my predictions (Fig. 7E & D, respectively). Both results might also be explained by the above-outlined interactions between the strain profile that was used, and the velocity increase that was associated with increasing frequency. Shortening-induced deactivation may also have been what contributed to the lack of differentiation among the maxima and actuation percentages of the three frequencies. Shortening-induced deactivation could have limited the maximum force and power output of this muscle (Josephson 1993; Askew & Marsh 1998; Ahn & Full 2002).
Though the change in actuation percentage was insignificant, it is worth examining some of its differences among the frequency treatments. The actuation percentage was likely highest at 6.1 Hz (Fig. 7A & F) because the slower velocity allowed more time for muscle contraction and force production to occur during the shortening phase without overlap with the lengthening phase (Josephson 1993; Askew & Marsh 1998; Ahn & Full 2002). That slower velocity also reduced power output, because it was probably not the muscle’s optimum shortening velocity, as was shown by the low power range at this frequency (Fig. 7A & E). Many of the phases likely exhibited such low positive power output that it was indistinguishable from net zero power, and therefore did not contribute to the actuation percentage. Increasing frequency resulted in net negative power output over a greater range of the stimulus onset phases, which was the reason why the actuation percentage, and mean power were lowest at the highest tested frequency: 10.1 Hz (Fig. 7C, D & F).

There was still a notable difference in the power output at -50 and 50% phase within a given treatment, however, this difference was dependant on the frequency treatment. Again, these differences could have occurred for the same reasons as were listed in the previous two sections, but varying frequency altered the degree to which these two phases differed from one another. At 6.1 Hz, the slower velocity allowed the muscle to produce force during the shortening phase, resulting in positive power (Josephson 1993; Askew & Marsh 1998). The slower velocity also reduced power output (Josephson 1993), which explains why the difference between the power output at -50 and 50% phase was subtler than at 8.6 or 10.1 Hz (Fig. 7A-C). In contrast, at the higher velocity 10.1 Hz treatment, the shortening phase was shorter and the muscle had less time to contract and produce force without it overlapping with the lengthening phase (Josephson 1993; Askew & Marsh 1998). The positive power output at -50% phase was dampened by this overlap, whereas negative power output at 50% phase was amplified by the increased overlap of force production with the lengthening phase (Fig. 7C).
2.4.4 Conclusions

Previous accounts of how changes in activation timing, activation duration and contractile cycle frequency can alter muscle performance, provide support for the results of this study. In the plantaris of bullfrogs (Lithobates catesbeianus) shifting the onset of activation produced a sinusoidal response curve (Sawicki et al. 2015). In vitro measures of the pectoralis muscles of both zebra finches (Taeniopygia guttata) and budgerigars (Melopsittacus undulatus) showed that when combined with changes in frequency and stimulus duration, subtle shifts in stimulus onset can generate significant changes in power output (Ellerby & Askew 2007). In two seemingly redundant leg muscles (177c and 179) of the cockroach (Blaberus discoioidalis) 177c generated positive power and 179 generated negative power under in vivo conditions, in part because of differences in their activation patterns (Ahn & Full 2002).

The results of this study in conjunction with the in vivo observations in the pigeon humerotriceps (Table 1), suggest that despite much of the variability in activation timing, duration and wingbeat frequency, this muscle is optimized as a brake when operating under in vivo conditions. This conclusion was derived from the predominance of negative power output across all the in vivo activation timings, regardless of wingbeat frequency or activation duration (Fig. 5-7, see Table 1 for in vivo measures). The sinusoidal strain profile and shortening-induced deactivation may have confounded the results by reducing force and consequently positive power output. However, deactivation of contractile elements has also been suggested to enhance muscle relaxation, and reduce the amount of work needed for lengthening, thereby increasing the net work and power output (Josephson & Stokes 1989). Therefore, it is unclear whether shortening-induced deactivation was a factor here.

The role of the pigeon humerotriceps that was inferred from the results of my study does not align with the previous hypothesis that this muscle serves primarily as an actuator to elbow extension and wing expansion (Dial 1992a; Robertson & Biewener 2012). The predominantly negative power output of this muscle indicates that it is absorbing energy to slow a motion (Josephson 1985; Josephson 1993; Dickinson
et al. 2000; Ahn & Full 2002; Sawicki et al. 2015). However, my results are potentially supportive of the hypothesis that the humerotriceps is used to stabilize the elbow joint angle during the transition from the upstroke to the downstroke (Dial 1992a; Robertson & Biewener 2012), since this would also require some degree of energy absorption. Additionally, certain stimulus phases within the biologically relevant range (Table 1) also caused the muscle to exhibit near net zero power output (Fig. 5-7), and the shapes many of the work loops are elongate, narrow and nearly vertical (Fig. 3). This suggests some level of resistance to lengthening, as well as varying degrees of muscle stiffness and elasticity (Tu & Dickinson 1994; Tu & Dickinson 1996; Roberts et al. 1997). The potential presence of these properties may be an indication of spring and strut-like behaviour (Tu & Dickinson 1996; Roberts et al. 1997).

Preliminary visual inspection of the EMGs that were recorded throughout the work loop experiments in this study suggest that maximally stimulating the pigeon humerotriceps by applying a train of square pulses to the brachial nerve may not accurately represent the in vivo activation patterns of this muscle. The EMG output showed that the train of muscle potentials were all very similar in height and width, not unlike the train of square pulses that was applied to the muscle and induced those potentials. This would be an underestimate of the true complexity of the neural activation patterns that underlie the in vivo muscle potentials that have been measured in the pigeon humerotriceps muscle (Dial 1992a; Robertson & Biewener 2012). However, these data have yet to be fully analysed, therefore it remains to be seen whether this muscle activation method is representative of what occurs in vivo.

Overall, my results suggest that there may in fact be more versatility and diversity in the way that the humerotriceps muscle can be used by pigeons to achieve fine motor control at the level of the elbow joint, than has been anticipated by previous studies (e.g.: Dial 1992a; Dial 1992b; Robertson & Biewener 2012).
3 Concluding Chapter

The purpose of this study was to use the in situ work loop technique (Nelson et al. 2004; Roberts & Azizi 2010) to characterize some aspects of the performance space of the pigeon humerotriceps. To do so, I examined how stimulus onset phase, stimulus duration, and contractile cycle frequency affect the power output of the humerotriceps muscle. My objective was to provide a better understanding of how this muscle is used to produce the fine motor control that pigeons display when actively modulating the shape of their wing (Dial 1992b; Robertson & Biewener 2012; Altshuler et al. 2015).

It is clear from the anatomy and in vivo measurements that the pigeon’s humerotriceps muscle helps to control some aspect of wing shape at the level of the elbow joint (Dial 1992a; Robertson & Biewener 2012). However, information about the humerotriceps muscle’s activation and strain patterns only provides a hint as to the actual role of this muscle in regulating the elbow joint angle (Dial 1992a; Ellerby & Askew 2007). Although the current study was not performed in vivo, the measures of work and power output obtained from the in situ work loop technique still provide a good idea of how a muscle might be functioning in vivo (Dickinson et al. 2000; Nelson et al. 2004; Roberts & Azizi 2010). The pigeon humerotriceps muscle exhibited negative power output over the majority of the stimulus onset phases, regardless of the treatment, which is highly suggestive that this muscle absorbs energy and serves primarily as a brake (Josephson 1985; Dickinson et al. 2000; Ahn & Full 2002; Sawicki et al. 2015).

Be it for terrestrial, aquatic or aerial locomotion, muscles are not simply motors that produce mechanical energy to move skeletal structures (Dickinson et al. 2000). Muscles necessarily perform fundamentally different functions ranging from actuator to brake, spring or strut, and they do so in a coordinated manner, that allows them to help produce locomotion (Josephson 1985; Roberts et al. 1997; Dickinson et al. 2000; Ahn & Full 2002; Sawicki et al. 2015).

Although muscles can be optimized to perform different roles, there are muscles that serve as motors by actuating motion, like the pectoralis muscles of birds for example, whose primary function is to power
flight by depressing and pronating the humerus to produce lift during the downstroke (Dial 1992a; Biewener et al. 1998; Ellerby & Askew 2007; Tobalske 2007; Robertson & Biewener 2012). Ellerby & Askew (2007) demonstrated that, in both zebra finches and budgerigars, the main method for modulating the power output of the pectoralis muscles across different flight speeds is by altering fascicle strain trajectory and motor unit recruitment. In this study, power output from the pectoralis muscle ranged from 41 to 111 W/kg in the zebra finch and 30 to 70 W/kg in the budgerigar (Ellerby & Askew 2007). In vivo measures in the pigeon’s pectoralis muscle showed that because the upstroke is more rapid than the downstroke, on average, the shortening phase of this muscle occupies 63% of its contractile cycle (Biewener et al. 1998). This is likely one reason why this muscle can produce such positive power; the mean in vivo power of the pigeon pectoralis muscle was 70.2 W/kg during slow, level flapping flight (Biewener et al. 1998).

As previously noted in chapter 2, one example of a muscle serving as a brake comes from a study of the two leg extensor muscles of cockroaches. These two muscles possess similar structure and twitch kinetics, but differences in their activation patterns and relative shortening velocities allowed one muscle to produce energy and the other to absorb it during locomotion (Ahn & Full 2002). The braking muscle (179) was much shorter than the actuator (177c), thus muscle 179 exhibited a relatively faster shortening velocity and likely operated closer to its maximum velocity than muscle 177c (Ahn & Full 2002). Muscle 179 also had a slightly longer in vivo activation duration than 177c (Ahn & Full 2002). As a result, under in vivo conditions, muscle 177c produced an average of 28.08±10.46 W/kg, whereas muscle 179 produced -19.13±14.09 W/kg (Ahn & Full 2002). The mechanical energy absorbed by muscle 179 is thought to slow flexion and stabilize the coxa-femur joint during locomotion (Ahn & Full 2002).

The first basalar muscles (b1) of the blowfly (Calliphora vicina) are thought to absorb work, for wing control during steering and maneuvering in flight (Tu & Dickinson 1994). Unlike most insect flight muscles, the b1 is activated by a single action potential that fires with every wingbeat, therefore this muscle’s output is modulated by phasic activation (Tu & Dickinson 1994). At lower frequencies (10 to 50 Hz), when
stimulus phase was just prior to muscle shortening, the b1 muscle produced positive power that was similar to what is produced in other insect flight muscles (Tu & Dickinson 1994). However, the in vivo wingbeat frequency of the blowfly is closer to 150 Hz, and with muscle activation occurring in parallel with this frequency, the net power output of the b1 muscle was always negative, regardless of activation phase or muscle strain (Tu & Dickinson 1994). The twitch kinetics of the b1 muscle likely prevented it from exhibiting net positive power output, since its twitch duration exceeded the length-change cycle period at a frequency that was well below that which is used in vivo (Tu & Dickinson 1994). Moreover, the magnitude of energy absorption in the b1 muscle was dependent on the activation phase, with phase shifts causing up to a 30% shift in work per cycle (Tu & Dickinson 1994). The phase modulation of the b1 muscle strongly suggests that the energy absorbed by this muscle is used to modulate wing supination for turning maneuvers (Tu & Dickinson 1994). Additionally, the elongate and narrow shape of the work loops that were produced by this muscle (Tu & Dickinson 1994) are highly suggestive that the b1 performs this function by serving as either a stiff or compliant spring (Tu & Dickinson 1996).

During running, in vivo force and length measures in the lateral gastrocnemius of wild turkeys suggest that this muscle serves primarily as a strut (Roberts et al. 1997). This muscle performs this role by producing most of its force when it is at a constant length, during the stance phase of running (Roberts et al. 1997). The stance phase is when the muscle must produce sufficient force to support the body and contribute work to lift and reaccelerate the animal (Roberts et al. 1997). Force and work done by the muscle is stored by tendon stretching (Roberts et al. 1997). The work done to lift and reaccelerate the animal comes from the spring-like behaviour of this muscle, where energy stored from the stance phase is released during muscle shortening and tendon recoil in the swing phase (Roberts et al. 1997). During the stance phase of level running, the work done by the muscle, and the work being done on the muscle (from the muscle contracting against a stretching tendon) are approximately equivalent and the resultant net work is nearly zero (Roberts et al. 1997). Furthermore, much of the tendon of the lateral
gastrocnemius becomes calcified and stiff in mature wild turkeys and the storage and release of elastic energy is thought to occur primarily in the tendon aponeurosis (Roberts et al. 1997). However, a considerable amount of this tendon (including the aponeurosis) do retain elasticity (Roberts et al. 1997). The reduced tendon length in the turkey’s lateral gastrocnemius is worth noting because the tendon of the pigeon humerotriceps muscle is quite short. Since this reduction in the tendon length of the turkey’s lateral gastrocnemius, through calcification, does not seem to prevent it from serving as a strut (Roberts et al. 1997), then the length of the pigeon’s humerotriceps tendon should not prevent it from performing this function either.

The *in vivo* measures of the activity patterns in the turkey’s lateral gastrocnemius also provide evidence that muscles undergo differential activation, depending on the type of behaviour or gait (Roberts et al. 1997). Intensity of muscle activation increases during gaits that require greater force production, such as inclined running (Roberts et al. 1997). Inclined running reduces force output of this muscle because it undergoes some shortening during the stance phase of this gait, but the force requirements are still met through increased muscle activation intensity and recruitment of more motor units (Roberts et al. 1997). Additionally, shifts in the length-change pattern of the turkey’s lateral gastrocnemius muscle can accommodate shift in work requirements for different locomotive behaviours (Roberts et al 1997; Gabaldón et al. 2004). During inclined running the function of this muscle can be shifted from strut to actuator, by increasing muscle shortening so that positive power output can occur (Roberts et al. 1997; Gabaldón et al. 2004). Conversely, reducing the amount of shortening allows this muscle to absorb work during declined running (Gabaldón et al. 2004). This type of functional modulation is also apparent in the peroneus longus, another ankle extensor, in wild turkeys (Gabaldón et al. 2004). However, the function of the peroneus longus is also modulated by changing activation pattern and shifting peak force production to earlier in the stance phase (Gabaldón et al. 2004). During the stance phase of inclined running, this muscle also produces net positive power, allowing the animal to meet energetic demands
from this gait and accelerate up the slope (Gabaldón et al. 2004). In contrast, when running downslope, the body of the animal must be decelerated, which would require energy absorption at the stance phase of running (Gabaldón et al. 2004). By shifting the length-change pattern of both the lateral gastrocnemius and the peroneus longus, and changing the activation pattern of the peroneus longus, these two muscles help to decelerate the body by producing net negative work and absorbing energy (Gabaldón et al. 2004). This study not only provides supporting evidence that shifts in muscle activation and length-change patterns are associated with changing gait and behaviour, but also provide support for the ability of muscles to function as brakes.

In these studies where muscles exhibited net negative work and power output and served to dissipate energy and decelerate motion, this function was exhibited in part because of long activation durations (Ahn & Full 2002), high shortening velocity (Tu & Dickinson 1994) or a shift in activation timing (Gabaldón et al. 2004). Therefore, these studies provide potential support for the results of the current study, which suggest that under the approximated in vivo activation durations, onset timings and cycle frequencies, the pigeon humerotriceps serves primarily as a brake. This suggests that the humerotriceps muscle is used to slow elbow flexion and wing folding from the upstroke, facilitating the transition to elbow extension and wing expansion for the downstroke during flight in pigeons.

There is also some evidence of spring and strut-like behaviour in the pigeon humerotriceps muscle, based on the near net zero power output at some of the stimulus phases (Fig. 5-7) that were within the biologically relevant range (Table 1). In addition to the lack of net work and power, the elongate and narrow shape of many of the work loops (Fig. 3) is suggestive of spring-like qualities (Tu & Dickinson 1994; Tu & Dickinson 1996). The near verticality of some of these loops (Fig. 3) suggested resistance to length change, and increased muscle stiffness (Tu & Dickinson 1994), which would be consistent with the isometric conditions under which strut-like behaviour usually occurs in a muscle (Roberts et al. 1997). These work loop features were present across many of the different phases and treatments. Muscle
stiffness was particularly prominent in work loops produced at negative stimulus onset phases, whereas the work loops exhibiting greater muscle compliance were more commonly produced at positive stimulus onset phases (Fig. 3B). This potential for spring- or strut-like function in the pigeon humerotriceps would support the hypothesis that this muscle is used to stabilize the elbow joint angle during the transition from wing folding during the upstroke to wing expansion during the downstroke (Dial 1992a; Robertson & Biewener 2012).

Finally, studies of muscle function during locomotion provide evidence that there can be a high level of variability in muscle activation pattern, which can ultimately provide some diversity in that muscle’s function based on the type of behaviour or the gait (Roberts et al. 1997; Gabaldón et al. 2004). Therefore, the high diversity in the in vivo measurements of the pigeon humerotriceps (Table 1) may be partially explained by this feature in muscles that has been previously documented during locomotion in other animals such as running turkeys (Roberts et al. 1997; Gabaldón et al. 2004).

The results of this study should be interpreted with caution; there are many different variables and parameters that can be manipulated during work loop experiments, which can ultimately affect the outcome of the study (Josephson 1985; Josephson 1993; Askew & Marsh 1998; Ahn & Full 2002; Sawicki et al. 2015). The pigeon’s humerotriceps muscle may in fact be more versatile in terms of its function than what is suggested by my current results, as well as what has been proposed by previous studies (Dial 1992a; Dial 1992b; Robertson & Biewener 2012). There are many inherent properties of a muscle, and external variables that can influence how that muscle is being used (Josephson 1985; Roberts et al. 1997; Dickinson et al. 2000; Ahn & Full 2002; Sawicki et al. 2015). Some of the limitations of the in situ work loop technique used for this study should therefore be taken into consideration.
3.1 Limitations

One of the biggest limitations of this study was my inability to replicate the *in vivo* strain profile. As was previously noted, it is not uncommon to use a sinusoidal strain trajectory as an approximation of the *in vivo* muscle strain profile, when examining muscle performance using the work loop technique (Josephson 1985; Josephson 1993; Askew & Marsh 1998; Sawicki et al. 2015). However, doing so involves making certain assumptions about the properties of the muscle that is being examined. In this case, it was assumed that the pigeon humerotriceps muscle’s strain profile could be approximated by a symmetrical sine wave strain pattern. As noted in the previous chapter, these assumptions may have caused underestimates of the positive power output and overestimates of negative power output (Josephson 1993). These results are further confounded when frequency is varied, and can cause an even greater bias towards negative power output (Josephson 1993; Askew & Marsh 1998; Ahn & Full 2002).

Another limitation of this study was that the applied stimulus pattern that I used during my work loop experiments may not have been an accurate representation of *in vivo* activation patterns. I have yet to determine how closely the EMG recordings from my work loop experiments resemble those recorded from the humerotriceps muscle *in vivo* during different flight behaviours (Dial 1992a; Robertson & Biewener 2012). It does, however, seem very likely that the train of square pulses that are used to stimulate the muscle during the work loop experiments do not effectively reproduce the complex neural activation pattern that is supplied to the muscle within a living animal. Again, changes in the activation pattern can greatly affect the power output and therefore alter the inferred role of a muscle (Josephson 1985; Ahn & Full 2002; Konow et al. 2015; Sawicki et al. 2015).

The experimental design also placed serious limitations on the longevity of the pigeon’s humerotriceps muscle. I chose to hold muscle temperature closer to its physiological temperature to obtain more biologically accurate measures of the force and power output from this muscle (Josephson 1985). However, in doing so, this caused force and muscle integrity to degrade more rapidly and limited
the number of treatments that could be tested during any experiment on an individual bird (Josephson 1985). In future studies of this nature, it may be worth considering the approach that Josephson (1985) used in his work loop studies, where the muscle was maintained at a lower temperature to increase its longevity and obtain a greater number of treatment measures from each subject.

Despite these limitations, the results of my study provide a better understanding of the function of the pigeon’s humerotriceps muscle. They also provide us with an appreciation for the complexity of the system that is involved in performing simple adjustments to the elbow joint angle, allowing these birds to actively modulate their wing shape and alter their flight behaviour using fine motor control. Although the story is incomplete, this information will still be valuable for bioinspired and biomimetic aircraft designs as it offers insight into the motor control system that birds use. This is important, since birds are capable of integrating visual guidance, sensory input, and motor control (Altshuler et al. 2015) effectively allowing them to outperform any manmade aircraft (Chin et al. 2017). Being able to apply knowledge of their muscle use for fine motor control, in conjunction with information about optimizing wing configuration and material properties of the wing structures could potentially help produce aircraft with better control, stability, and maneuverability (Chin et al. 2017).

**3.2 Future directions**

The *in situ* work loop technique has great potential for providing further insight into the way that birds use their intrinsic wing muscles to actively modulate their wing shape. Future studies that employ this technique would be greatly benefitted by researchers performing their own *in vivo* measures on their study subjects prior to taking work loop measures. These *in vivo* measures could then be used as parametric settings for the work loop experiments, and would be more reflective of the conditions that the muscle of interest would be subjected to within a given study subject (Josephson 1985; Josephson 1993; Sawicki et al. 2015).
Measures would ideally include EMG recordings to determine the muscle activation duration, pattern, and onset timing (Dial 1992a; Dial 1992b; Robertson & Biewener 2012). Simultaneous measures of the muscles contractile cycle and strain profile (i.e.: amplitude, trajectory and lengthening-shortening phase ratio) relative to the wingbeat cycle could be achieved using sonomicrometry and kinematics (Berg & Biewener 2008; Berg & Biewener 2010; Robertson & Biewener 2012). The greatest challenge with executing this technique will be the ability to then program the work loop parameters using these data.

Repeating the current study using the above-outlined adjustments would allow for more accurate inferences of the muscle’s function based on the measures of its net work and power output. Once this has been achieved, it would be worth using these same methods to examine the function of the biceps and scapulotriceps. Characterizing the performance space of all three of these muscles would provide a clearer account of the way that birds actively modulate the shape of their wing, since this set of muscles is thought to be responsible for regulating the elbow joint angle (Dial 1992a; Robertson & Biewener 2012).
References


Bahlman, J. W. and Altshuler, D. L. *(In review)*. Muscle power output explains the preferred wing kinematic strategies of small birds. eLife Sciences.


