THESIS

THE METABOLIC PROFILE OF CARDIAC AND SKELETAL MUSCLE TISSUE FROM CAPTIVE EMUS (*DROMAIUS NOVAEHOLLANDIAE*) RAISED IN NORTHERN COLORADO: IMPLICATIONS FOR ASSESSING MUSCLE HEALTH FOR EMUS WITH SPLAYED-LEG DISORDER

Submitted by

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ABSTRACT

THE METABOLIC PROFILE OF CARDIAC AND SKELETAL MUSCLE TISSUE FROM CAPTIVE EMUS (*DROMAIUS NOVAEHOLLANDIAE*) RAISED IN NORTHERN COLORADO: IMPLICATIONS FOR ASSESSING MUSCLE HEALTH FOR EMUS WITH SPLAYED-LEG DISORDER

Emus (*Dromaius novaehollandiae*) are native Australian flightless birds commonly farmed in North America for oil and meat. The emu industry has grown in popularity, though the physiology of farm-raised emus has not been studied extensively. Emus are efficient at both anaerobic sprinting and aerobic, sustained running, though it is unclear if they are inherently predisposed to be terrestrial athletes. Though some wild emu energetic studies have been completed, few studies have been performed on domesticated emu skeletal muscle, and there have been no investigations into the enzymatic profile of domesticated ratite (group of paleognathous flightless birds that includes emus) cardiac tissue.

This thesis established the first ratite cardiac metabolic profile by determining the myoglobin concentration (3.41 ± 0.06 mg g^{-1} wet tissue) and activities of citrate synthase (CS 19.4 ± 1.2 μmol min^{-1} g^{-1} wet tissue), β-hydroxyacyl CoA dehydrogenase (HAD 78.9 ± 3.6 μmol min^{-1} g^{-1} wet tissue), and lactate dehydrogenase (LDH 239.5 ± 8.9 μmol min^{-1} g^{-1} wet tissue) of domesticated emu cardiac tissue. Emus had ~22% higher myoglobin levels in cardiac tissue compared to domesticated chickens, and ~89% higher that of domesticated dogs. The metabolic potential of emu cardiac tissue appeared more similar to that of active flying birds, such as pigeons and geese. Enzymatic characteristics of the heart illustrate domesticated

emus can be metabolically categorized with other animals classified as elite avian and mammalian athletes.

This study also sets a metabolic baseline for three pelvic limb skeletal muscles (control limb): *M. gastrocnemius medialis* (GM) – myoglobin 2.74 ± 0.03 mg g^{-1} wet tissue, citrate synthase (CS) 12.14 ± 1.21 µmol min^{-1} g^{-1} wet tissue, β-hydroxyacyl CoA dehydrogenase (HAD) 14.05 ± 0.52 µmol min^{-1} g^{-1} wet tissue, lactate dehydrogenase (LDH) 905.44 ± 67.58 µmol min^{-1} g^{-1} wet tissue; *M. iliofibularis* (IFB) – myoglobin 3.04 ± 0.08 mg g^{-1} wet tissue, CS 18.43 ± 0.85 µmol min^{-1} g^{-1} wet tissue, HAD 23.04 ± 2.28 µmol min^{-1} g^{-1} wet tissue, LDH 913.08 ± 57.92 µmol min^{-1} g^{-1} wet tissue; *M. iliofemoralis externus* (IFME) – myoglobin 4.43 ± 0.07 mg g^{-1} wet tissue, CS 18.23 ± 0.40 µmol min^{-1} g^{-1} wet tissue, HAD 28.66 ± 0.91 µmol min^{-1} g^{-1} wet tissue, LDH 947.65 ± 37.70 µmol min^{-1} g^{-1} wet tissue. Additionally, for future studies on ratite orthopedic disorders, metabolic profiles of three pelvic muscles from both limbs (splayed limb and non-splayed limb) of emus affected by splayed-leg disorder were recorded. In general, both limbs of the splayed-leg emus had higher levels of metabolic proteins, and therefore looked more athletic than the control limbs. Though these emus had an orthopedic disorder, it was found their muscles increased in aerobic capacity, as evidenced by enhanced enzyme activities and myoglobin levels to offset the additional workload caused by the disorder.

ACKNOWLEDGEMENTS

I am extremely grateful to Linn E. Turner and Terry A. Turner (Rabbit Creek Emu Ranch) for allowing us access to their facilities and emus, as well as supplying numerous resources to us for this project. I thank the Grin and Barrett Emu Ranch (in memory of Lola Barrett) for permitting samples to be taken from their emus, and Wayne M. Hanson and workers at Wind River Processing Inc. for their helpfulness and expertise during the collection process. The American Emu Association (AEA) Board of Directors supplied information regarding approximate AEA emu ranch localities in the United States. Dr. Shane B. Kanatous has been an extremely loyal and influential advisor, and his guidance is irreplaceable. I would also like to thank Michael A. De Miranda Jr., Ashley M. Larson, Amber E. Schlater, Caitlin E. Kielhorn, and all other members of the Kanatous Lab for their assistance with this project, as well as the Department of Biology and Department of Biomedical Sciences at Colorado State University. Funding was provided to Dr. Shane Kanatous from Colorado State University.

AUTOBIOGRAPHY

I was in born Denver and raised by my parents Richard and Jane Green. From a young age I became fascinated with the natural world. At the age of nine, my interests in zoology and geology drove me to join the Western Interior Paleontological Society. In this professional society I became familiar with field techniques, presentation styles, and the scientific method. This passion continued through my undergraduate degree at Colorado State University. As an undergraduate, I completed an independent study with Brent Breithaupt from the University of Wyoming. During this study I became familiar with emus, conducting a growth study by tracking measurements of emus from the time they hatched to adulthood. These data were used to make comparisons between emu development, and the development of Jurassic theropod dinosaurs. During this project, I was exposed to orthopedic disorders that some of the emus possessed, and became more interested in the physiology. As I joined Dr. Shane Kanatous' lab at Colorado State University the combination between his enthusiasm in comparative biology, and Linn and Terry Turner's hospitality at the Rabbit Creek Emu Ranch, really made this project hit the ground running. This degree has been an amazing opportunity in my life, and I appreciated every second.

DEDICATION

My Master's thesis is dedicated first and foremost to by parents, Richard E. Green and Jane R. Green. Without their unyielding support and inspiration, the passions in my life may have never been explored. Linn E. Turner, Terry A. Turner, and Brent H. Breithaupt allowed me to live out a dream, full of giant birds. Dr. Shane B. Kanatous, my labmates, friends, and family have made graduate school unforgettable, and I an eternally grateful to all of them.

-- T.L.G

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CHAPTER 1

Introduction

Paleognathous birds are the small avian lineage that includes tinamous (*Tinamus, Nothocercus, Crypturellus, Rhynchotus, Nothoprocta, Nothura, Taoniscus, Eudromia,* and *Tinamotis*), rheas (*Rhea*), kiwis (*Apteryx*), ostriches (*Struthio*), emus (*Dromaius*), and cassowaries (*Casuarius*). Due to a similar palate structure, the flighted tinamous are considered paleognaths, though ratites are specifically the paleognathous birds that are fully flightless (Kaiser, 2007). Emus (*Dromaius novaehollandiae*, Latham 1790) are the second largest (45 kg) ratite species, native to Australia, though they were imported to North America as exotic pets. Although Australian Aborigines began harvesting emus centuries ago, it was not until the 1980's when Americans began to notice the marketable value, and began raising emus as livestock animals for oil, meat, leather, eggs, and feathers (Minnaar, 1998). Currently, over one million emus are ranched throughout the United States for commercial products (American Emu Association, 2009). Emus can tolerate extremes in temperature, sustaining a constant body temperature of approximately 39°C in ambient conditions ranging from -5 to 45°C (Jones et al., 1983; Maloney and Dawson, 1994), which is why domesticated populations can persist throughout the United States. Emu ranches are even found in cold regions of northern Europe and Canada (Minnaar, 1998). Adult emus are usually kept outside year round, including the breeding season. For emus from the northern hemisphere, the breeding season occurs November through April, during the cold winter months (Minnaar, 1998). Emus in their native southern hemisphere will breed during the rainy winter months of May through October (Minnaar, 1998).

Ratites are unique model organisms for physiological and anatomical studies because they are exceptional athletes. Ostriches are the fastest terrestrial bird, reaching speeds of 70 km

hr^{-1} (Saunders and Fedde, 1994). Emus can sprint up to 50 km hr^{-1} (O'Brien, 1990) and can run for long distances without stopping. Though emus and other ratites have unique athletic potential, little cardiac physiology has been described in this taxonomic group of birds. Many of the physiological experiments involving emus have focused on exercise responses and limb kinematics (Grubb et al., 1983; Maloney, 1994; Claessens, 2009). Emus, trained to run on treadmills, have exhibited an 11.4-fold increase in oxygen consumption, 3.9-fold increase in heart rate, and 1.8-fold increase in stroke volume from rest to high-intensity exercise (Grubb et al., 1983). These findings suggest emus have the capacity to be elite athletes, though it is unknown if their tissue is molecularly predisposed to be athletic.

Cardiac tissue, because it contracts continuously, relies predominantly on fat oxidation and aerobic metabolism (Katz, 2006). However, the magnitude of cardiac aerobic reliance is dependent upon the enzymes and proteins that make up the tissue. For example, tufted ducks (*Aythya fuligula*) are highly athletic migratory birds, and therefore have higher levels of aerobic proteins in cardiac tissue compared to less active birds (Saunders and Fedde, 1994). Though untrained, captive tufted ducks have significantly lower levels of citrate synthase (CS; an aerobic marker protein) in cardiac tissue compared to naturally trained birds, these untrained birds still have relatively higher activities of aerobic enzymes compared to less aerobically-active bird species, like the domesticated chicken (Alp et al., 1976; Butler and Turner, 1988). It is likely that there is an inherent component in cardiac tissue that contributes to elite athleticism in birds. One of the goals was to investigate cardiac muscle proteins to determine whether emus are inherently suited for high levels of activity by possessing high cardiac myoglobin and metabolic enzyme activity.

Historically, avian cardiac physiology was studied in diving birds and small domesticated farm birds. More recently, migrating bird physiology has become of greater concern. Long-distance migrating birds are especially interesting to researchers because they have the ability to sustain high-intensity exercise for extended periods of time. For example, golden plover (*Pluvialis dominica*) undergo a non-stop migration 3,800 km long (Henshaw, 1910) and migratory bar-tailed godwits (*Limosa lapponica baueri*) have been observed flying for approximately eight straight days, 10,000 km southward across the Pacific Ocean (Gill et al., 2009). Bar-headed geese (*Anser indicus*) not only undergo long migrations, but they do so over the Himalaya Mountains at about 9,000 m above sea level (Butler, 2010; Swan, 1970). The cardiac tissue of bar-headed geese reflects this athletic ability, in having high levels of the aerobic protein, myoglobin (6.0 mg g^{-1} wet tissue), compared to less-active birds, like the chicken (2.8 mg g^{-1} wet tissue) (Nishida, 1976; Saunders and Fedde, 1991). Many birds have the ability to sustain high-intensity exercise for an extended period of time. Terrestrial birds are no exception – the ostrich (*Struthio camelus*) can maintain speeds of 40 km hr^{-1} for over 30 minutes (Saunders and Fedde, 1994). In comparison, humans (*Homo sapiens*) have been known to reach speeds of 40 km hr^{-1}, though can only sustain such high speeds for less than a minute (Olympic.org). In addition, it is unknown if metabolic characteristics of cardiac tissue from flightless birds, like the emu and ostrich, would be physiologically similar to those of flying birds.

Being strictly terrestrial birds, emus rely on different locomotory means than flying birds. Compared to other terrestrial birds, ratites have greater overall mass in their hindlimbs (Patak, 1988). The hindlimb muscles, which emus use for propulsion, account for approximately 25% of the overall weight of the bird (Patak, 1988). The ostrich (*Struthio camelus*), another ratite

species, has hindlimb muscles that contribute about 34% of the overall body mass (Smith et al., 2006). In contrast, the emperor penguin (*Aptenodytes forsteri*) pelvic limb musculature makes up only 6-7% of their overall mass (Ponganis et al., 1997), and the muscles and bones in the hindlimbs of the domesticated chicken (*Gallus gallus domesticus*) make up only 20% of the overall body mass. Ratites have an even greater percentage of mass in their pelvic limb muscles compared to humans (*Homo sapiens*), 17-20% (Janssen et al., 2000); a mammal that relies solely on its hindlimbs for propulsion. The percentage of pelvic limb muscle mass of ratites (25%) is similar to the percent pectoral muscle mass contributes to overall body mass in flying birds (10–35%) (Hartman, 1961; Patak, 1988; Patak and Baldwin, 1993).

Because ratites exhibit high-speed running, the hindlimb anatomies of emus (Patak and Baldwin, 1993), ostriches (Smith et al., 2006), and rheas (Piasso, 2010) have been examined and classified to aid in the understanding of ratite biomechanics. Many of the previous skeletal muscle studies have been conducted on wild Australian emus rather than domesticated emus. The metabolic profile and fiber typing of a variety of pelvic limb skeletal muscles were determined from emus from Western Victoria, Australia in 1993 (Patak and Baldwin, 1993). The data collected from these techniques suggested that emu pelvic limb skeletal muscles were similar in metabolic makeup (highly aerobic) to pectoral muscles of flying birds (Patak and Baldwin, 1993). Although limited data on emu exercise physiology exists, a sufficient account of healthy domesticated emu physiology has never been compiled. The second aim of the study was to set a metabolic baseline for domesticated emu skeletal muscle.

The magnitude of aerobic reliance is dependent on whether an animal is inherently predisposed for sprinting (anaerobic) or endurance (aerobic) activity. For example, predatory animals may depend more upon anaerobic metabolism in order to fuel fast bursts of speed, while

the muscles of prey animals may be fueled more by aerobic metabolism (Lindstedt, 1991; Williams et al., 1997). Although this predator-prey model is a wonderful example, the amount of aerobic and anaerobic metabolism an animal relies on during exercise may be dependent on numerous factors. An animal's environment (Kooyman and Ponganis, 1998; Kanatous et al., 1999) and body mass (Jones and Lindstedt, 1993) can play a major role in influencing the metabolic profile. Animals can accomplish elite athletic performance regardless of which metabolic system they use more for activity. The African cheetah (*Acinonyx jubatus*), a fast terrestrial runner that can reach speeds of up to 103 km hr^{-1} (Sharp, 1997), relies mostly on anaerobic metabolism for sprinting (Williams et al., 1997). In contrast, pronghorn (*Antilocapra americana*) can reach alleged speeds of 100 km hr^{-1} while relying on aerobic metabolism for endurance running (Lindstedt, 1991). This study will investigate muscle proteins in domesticated emus to determine whether specific pelvic limb skeletal muscles are more inherently suited for anaerobic or aerobic activity. The metabolic profile of domesticated emu skeletal muscle had never been identified, and it is necessary for determining a baseline for healthy animals. The baseline metabolic profile will also allow for comparative studies between healthy and pathologic domesticated emus.

Hindlimb orthopedic disorders (especially trauma-related) are fairly common in domesticated ratites on farms and in zoos (Gnad et al., 1996; Minnaar, 1998; Rothschild and Rühli, 2007; Cooper et al., 2008), and these disorders have been of interest to emu caretakers. A specific condition called splayed-leg disorder is especially prevalent in farm-raised emus, and is a growing concern. The cause of splayed-leg disorder is thought to be multifactorial (Gnad et al., 1996); low genetic diversity, poor nutrition, lack of exercise, and injuries are thought to be possible causes for ratite splayed-leg disorder (Gnad et al., 1996; Jenkins, 1996). Although

studies have identified osteologic disorders in ratites (Rothschild and Rühli, 2007), it is unknown whether defective bones cause muscles to become weak, or if defective muscles cause bones to twist. Additionally, this study measured the metabolic properties in muscle tissue from a splayed-leg individual to see if differences exist between it and its healthy counterparts.

To our knowledge, the metabolic profile of cardiac tissue has never been determined from wild or domesticated ratites. The metabolic profile from wild emu skeletal muscle has been determined, though a similar study with domesticated emus had not. The objective was to classify the metabolic properties of emu cardiac and skeletal muscle by measuring myoglobin concentrations and enzymatic activities. Data from this study set baseline metabolic profiles for domesticated emus for future studies. Metabolic data collected from this study also illustrate where the emu, a strictly terrestrial bird, fits into the spectrum of avian athletes. This study will also allow comparisons to be made between healthy emus, and ones with orthopedic disorders. Emu ranching is still a popular business in the United States, and as products become more popular, there is a high demand from emu ranchers and the general public to continue raising healthy birds. Understanding the physiological capabilities of healthy emus will further help to determine how disease and environmental factors affect the growth and development of emus in North America.

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CHAPTER 2

Hearts of a feather? Classifying the metabolic profile of cardiac tissue from farm-raised emus (*Dromaius novaehollandiae*) from northern Colorado

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Introduction

Ratites are the group of paleognathous flightless birds that includes rheas (*Rhea*), kiwis (*Apteryx*), ostriches (*Struthio*), emus (*Dromaius*), and cassowaries (*Casuarius*) (Fig. 2.1). Emus (*Dromaius novaehollandiae*, Latham 1790) are a large (45 kg) ratite species native to Australia, though they are commonly raised in North America as livestock animals, mostly for oil, meat, and leather. Currently, over one million emus are raised in the United States for commercial products (American Emu Association, 2009).

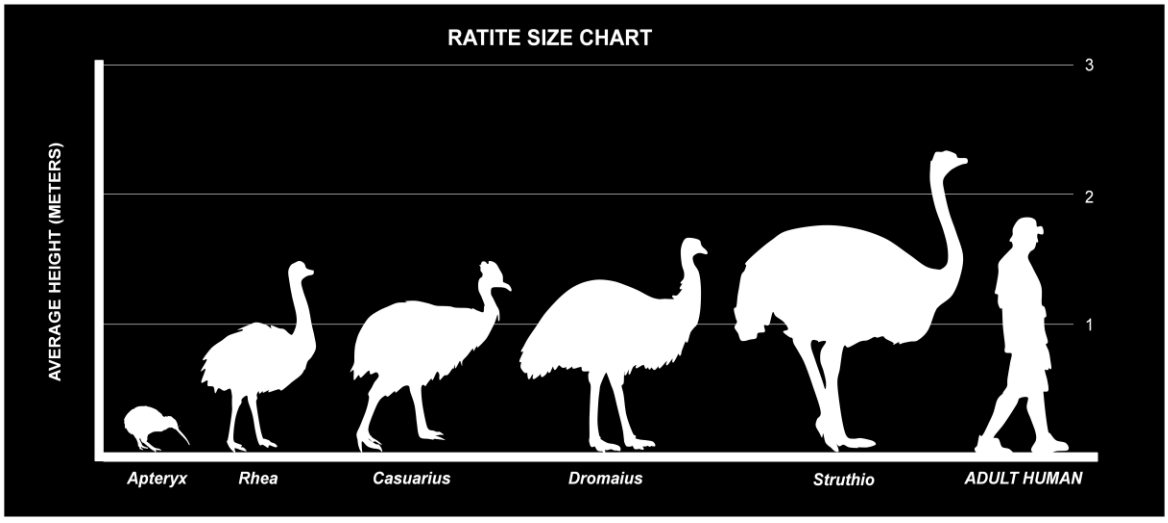

Fig. 2.1. Extant ratites (the flightless group of paleognathous birds) include kiwis (*Apteryx*), rheas (*Rhea*), cassowaries (*Casuarius*), emus (*Dromaius*), and ostriches (*Struthio*). Average height of ratites and human expressed in meters (m). (Illustration by Todd L. Green)

Emus can tolerate extremes in temperature, sustaining a constant body temperature of approximately 39°C in ambient conditions ranging from -5 to 45°C (Jones et al., 1983; Maloney and Dawson, 1994), which is why domesticated populations can persist

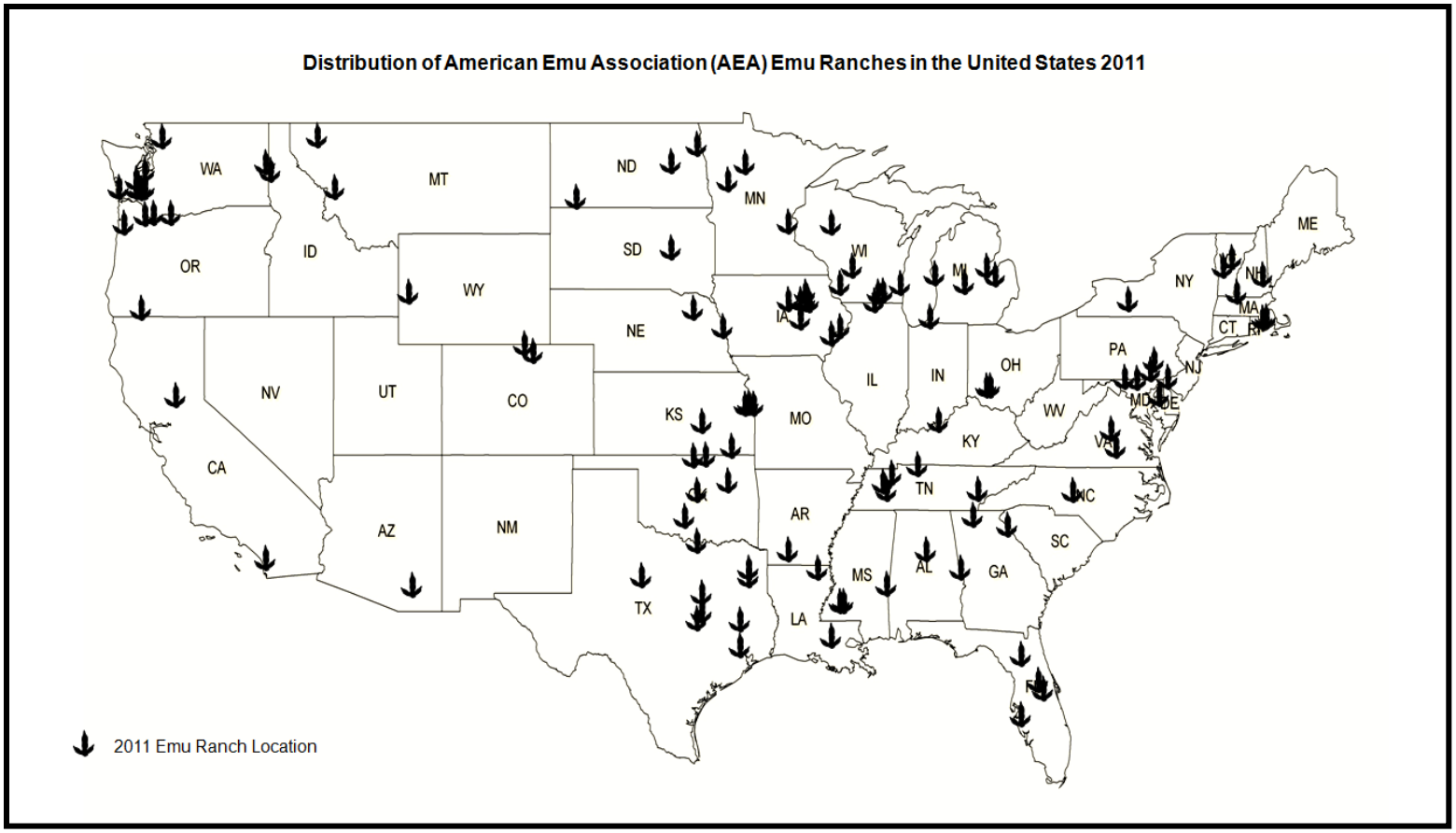

Fig. 2.2. This distribution map of domesticated emu ranches throughout the United States illustrates emus can survive in a variety of climates. Each emu footprint on this map represents an emu ranch affiliated with the American Emu Association (AEA). However, emus are also found in many other localities in North America. (Illustration by Todd L. Green, data supplied by the American Emu Association)

throughout the United States (Fig. 2.2). Emus are even farmed in the cold regions of northern Europe and Canada (Minnaar, 1998).

Emus, and other ratites, are strong terrestrial runners. Being strictly terrestrial birds, emus rely on different locomotory means than flying birds. Compared to other terrestrial birds, ratites have greater overall mass in their hindlimbs. The hindlimb muscles, which emus use for propulsion, account for approximately 25% of the overall weight of the bird (Patak, 1988). The ostrich (*Struthio camelus*), another ratite species, has hindlimb muscles that contribute about 34% of the overall body mass (Smith et al., 2006). In contrast, the emperor penguin (*Aptenodytes forsteri*) pelvic musculature makes up only 6-7% of their overall mass (Ponganis et al., 1997), and the muscles and bones in the hindlimbs of the domesticated chicken (*Gallus gallus domesticus*) make up only 20% of the overall body mass. Ratites have an even greater percentage of mass in their pelvic muscles compared to humans (*Homo sapiens*), 17-20% (Janssen et al., 2000); a mammal that relies solely on its hindlimbs for propulsion. The percentage of pelvic muscle mass of ratites is similar to the percent pectoral muscle mass contributes to overall body mass in flighted birds (Hartman, 1961; Patak, 1988; Patak and Baldwin, 1993).

These muscular hindlimbs allow emus to reach speeds of up to 50 km hr^{-1} (O'Brien, 1990), and also to undergo long periods of endurance running. Because emus exhibit high-speed running, past studies have examined pelvic muscles of wild emus and analyzed fiber type, histochemical properties, and muscle function (Patak and Baldwin, 1993; Patak and Baldwin, 1998; Main and Biewener, 2007). Many of the physiological experiments involving emus have focused on exercise responses and kinematics (Grubb et al., 1983; Maloney et al., 1994; Claessens, 2009). Emus, trained to run on treadmills, have exhibited an 11.4-fold increase in

oxygen consumption, 3.9-fold increase in heart rate, and 1.8-fold increase in stroke volume from rest to high-intensity exercise (Grubb et al., 1983). These findings suggest that emus have the capacity to be elite athletes, though it is unknown if they are molecularly predisposed to be athletes. Although limited data on emu exercise physiology exists, a sufficient account of healthy domesticated emu physiology has never been compiled. Specifically, little cardiac physiology has been described in ratites.

Because it contracts continuously, cardiac tissue relies greatly upon aerobic metabolism, which fat oxidation commonly fuels (Katz, 2006). However, the magnitude of aerobic reliance is dependent upon whether an animal is inherently predisposed for elite athletic performance. For example, tufted ducks (*Aythya fuligula*) are highly athletic migratory birds, and therefore have higher levels of aerobic proteins in cardiac tissue compared to less active birds (Saunders and Fedde, 1994). Though untrained, captive tufted ducks have significantly lower levels of citrate synthase (CS; an aerobic marker protein) in cardiac tissue compared to naturally trained birds, these untrained birds still have relatively higher levels of aerobic proteins compared to less aerobically-active bird species, like the domesticated chicken (Alp et al., 1976; Butler and Turner, 1988). It is likely that there is an inherent component to elite athleticism in birds. The goal was to investigate cardiac muscle proteins to determine whether emus are inherently suited for high levels of activity.

Historically, avian cardiac physiology was studied in diving birds and small domesticated farm birds. More recently, migrating bird physiology has become of greater concern. Long-distance migrating birds are especially interesting to researchers because they have the ability to sustain high-intensity exercise for extended periods of time. For example, golden plover (*Pluvialis dominica*) undergo a non-stop migration 3,800 km long (Henshaw, 1910), and

migratory bar-tailed godwits (*Limosa lapponica baueri*) have been observed flying for approximately eight straight days, 10,000 km southward across the Pacific Ocean (Gill et al., 2009). Bar-headed geese (*Anser indicus*) not only undergo long migrations, but they do so over the Himalaya Mountains at about 9,000 m above sea level (Butler, 2010; Swan, 1970). The cardiac tissue of bar-headed geese reflects this athletic ability, in having high levels of the aerobic protein, myoglobin (6.0 mg g^{-1} wet tissue), compared to less-active birds, like the chicken (2.8 mg g^{-1} wet tissue) (Nishida, 1976; Saunders and Fedde, 1991). Many birds have the ability to sustain high-intensity exercise for an extended period of time. Terrestrial birds are no exception – the ostrich (*Struthio camelus*) can maintain speeds of 40 km hr^{-1} for over 30 minutes (Saunders and Fedde, 1994). It is unknown if metabolic characteristics of cardiac tissue from flightless birds, like the emu and ostrich, look similar to those of flighted birds.

To our knowledge, the metabolic profile of cardiac tissue has never been determined from wild or domesticated ratites. The objective was to classify the metabolic properties of emu cardiac tissue by measuring myoglobin concentrations and enzymatic activities. Data from this study set a baseline cardiac metabolic profile for future ratite studies. Metabolic data collected from this study also illustrate where the emu, a strictly terrestrial bird, fits into spectrum of avian athletes. Based on behavioral data, emus appear to be good terrestrial athletes, thus it was expected that emu cardiac tissue would possess similar aerobic potential to other vertebrates that exhibit high levels of endurance activity. The total population of emus in the United States continues to grow as products become more popular. There is a high demand from emu ranchers and the general public to raise healthy birds for quality products. Understanding the physiological capabilities of healthy emus will further help to determine how disease and environmental factors affect the growth and development of emus in North America.

Materials and methods

Study animals

Heart samples were collected from emus raised on two ranches in northern Colorado, USA. Emus from the Rabbit Creek Emu Ranch (*n*=4), in Livermore, Colorado, USA lived in an outdoor pen at an elevation of 2,042 m (6,700 ft), while emus from the Grin and Barrett Emu Ranch (*n*=8), in Laporte, Colorado, USA lived in an outdoor pen at an elevation of 1,562 m (5,125 ft). Of the 12 emus, 10 were sub-adults (between 1-2 years old) and one was a breeding adult female (>4 years old) (Fig. 2.3). Age classifications used for this study were based upon physical and behavioral indications of sexual maturity and breeding status of the emus (T. L. Green, unpublished results). Two of the sub-adult birds had a splayed-leg disorder, an orthopedic disorder where one limb twists out laterally from the body. Although the limited sample size did not allow for statistical analysis on these individuals, we looked for trends in the data between the splayed and non-splayed birds for future studies. Heart samples were collected from the left ventricle, after emus from both sites were butchered by employees at Wind River Processing Inc. (Thermopolis, WY, USA) on March 15, 2010. These samples were immediately wrapped in aluminum foil and placed in a charged liquid nitrogen dewar, until they could be stored in a -80°C freezer at Colorado State University for later analysis of metabolic characteristics. Adult Weddell seal (*Leptonychotes weddellii*) skeletal muscle samples, originally collected in October 2006 by S. B. Kanatous near McMurdo Sound, Antarctica. These readily available samples were used as internal controls for all assays.

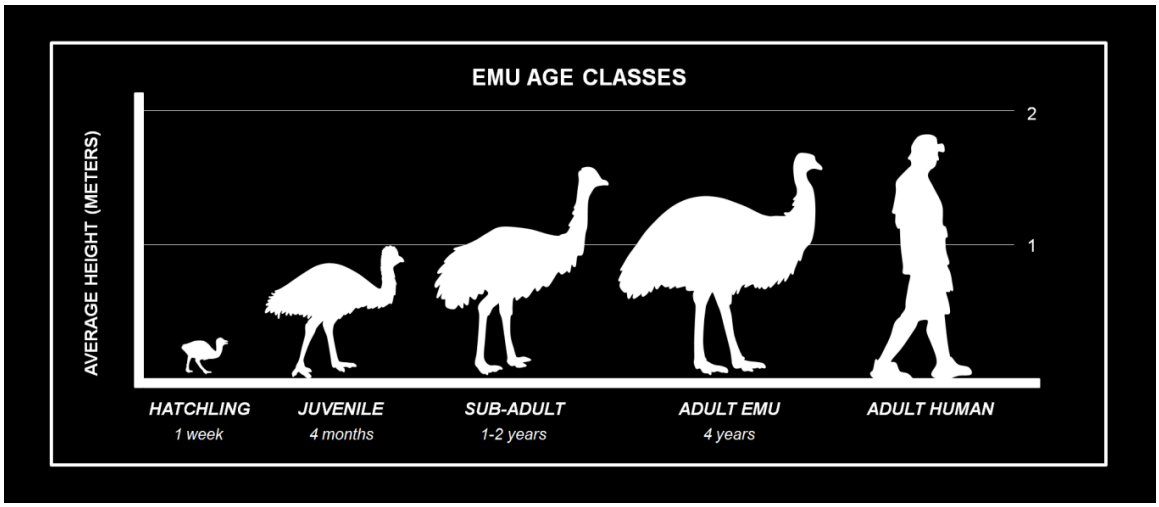

Fig. 2.3. Emus are the second largest living bird. During development, most of their mass is gained in the first year. Emus have been used as modern analogs for theropod dinosaur growth and development. For the present study, emus that were between 1-2 years of age were classified as "sub-adults" and emus > 4 years of age were classified as "adults". Average height of emus and human expressed in meters (m). (Illustration by Todd L. Green)

Myoglobin assays

The concentration of myoglobin, an oxygen-binding heme protein, was determined for the cardiac tissue. For all myoglobin and enzyme assays the cardiac tissue was homogenized with a hand glass homogenizer. A homogenization buffer was made with 79% PBS (phosphate buffered saline), 1% Tween-20, 20% glycerol, 0.001M DTT (dithiothreitol), and a protease inhibitor tablet (1/10 µl). 250 mg of tissue was weighed and homogenized (0°C), and the original dilution recorded. The resulting homogenates were clarified in a centrifuge at 10,000 x ***g*** for 10 min. The supernatant was collected, aliquoted, and stored in a -80°C freezer until it was used for the assays. Protein concentrations were determined for all emu cardiac samples by using Pierce Coomassie Plus Protein Assay Reagent (Pierce Chemicals, Rockford, IL, USA) before enzymatic assays were performed. Myoglobin assays were performed in accordance with the Reynafarje (1963) method adapted by Kanatous et al., 2002 with the following modifications: a portion (100 µl) of the homogenate was further diluted with 650 µl of phosphate buffer (0.04 mol l^{-1}, pH 6.6). The resulting mixture was centrifuged for 50 min at 28,000 x ***g*** at

4°C. The supernatant was bubbled with 99.9% carbon monoxide for 3 min. Spectrophotometric absorbance was measured at 538 nm and 568 nm, and the concentration of myoglobin in mg g^{-1} wet mass of muscle was calculated as: (Abs_{538}–Abs_{568}) X 5.865 [(1.5/0.5) X (dilution of sample)].

Enzyme assays

Enzymatic activity of citrate synthase (CS–aerobic potential), beta-hydroxyacyl CoA dehydrogenase (HAD–aerobic, β-oxidation), and lactate dehydrogenase (LDH–anaerobic potential) were determined for the cardiac tissue samples. The assay conditions were set according to previous studies (Reed et al., 1994; Kanatous et al., 2002): *Citrate synthase* (CS; EC 4.1.3.7): 50 mmol l^{-1} imidazole; 0.25 mmol l^{-1} 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB); 0.4 $mmol^{-1}$ acetyl CoA; and 0.5 mmol l^{-1} oxaloacetate, pH 7.5 at 37°C; ΔA_{412}, ε_{412}=13.6. *β-hydroxyacyl CoA dehydrogenase* (HAD; EC 1.1.1.35): 50 mmol l^{-1} imidazole; 1 mmol l^{-1} EDTA; 0.1 mmol l^{-1} acetoacetyl CoA; and 0.15 mmol l^{-1} NADH, pH 7.0 at 37°C; ΔA_{340}, ε_{340}=6.22. *Lactate dehydrogenase* (LDH; EC 1.1.1.27): 50 mmol l^{-1} imidazole; 0.15 mmol l^{-1} NADH, pH 7.0 at 37°C; and 1 mmol l^{-1} pyruvate; ΔA_{340}, millimolar extinction coefficient ε_{340}=6.22. Myoglobin and enzyme assays were determined by using a BioTek Synergy HT Multi-detection microplate reader (Winooski, VT, USA). Specific enzyme activities (μmol min^{-1} g^{-1} wet mass of muscle) were calculated from the rate of change of the assay absorbance at the maximal linear slope and normalized to wet tissue weight. The CS:HAD ratio was calculated in order to estimate the dependence of aerobic metabolism upon lipid oxidation.

Statistical analyses

All muscle samples were run as triplicates, and each assay was repeated three times. Skeletal muscle samples from an adult Weddell seal were used as controls for the assays. Data from these two emu ranches were pooled into one data set for the determination of cardiac profile (Table 2.1). The statistical analyses were done *via* SigmaStat 2.0 (Ashburn, VA, USA). Analysis of variance (one-way ANOVA) with Tukey *post-hoc* tests were used ($P \leq 0.05$), and the results given as means ± S.E.M.

Results

The emus from this study had an average myoglobin concentration of 3.41 ± 0.06 mg g^{-1} wet tissue (Table 2.1). Average CS activity in the cardiac tissue was 19.4 ± 1.2

Table 2.1. Metabolic Profile of Emu Cardiac Tissue

Organism	Cardiac Myoglobin	Cardiac CS	Cardiac HAD	Cardiac CS:HAD	Cardiac LDH
Domesticated Emu	3.41 ± 0.06	19.4 ± 1.2	78.9 ± 3.6	0.25	239.5 ± 8.9

Cardiac profile of domesticated emus raised in Laporte and Livermore, Colorado. n=12
Myoglobin concentrations are presented as mg g^{-1} wet tissue; enzyme activities (CS=citrate synthase; HAD= β-hydroxyacyl CoA dehydrogenase; LDH=lactate dehydrogenase) are presented as μmol min^{-1} g^{-1} wet tissue.
Means ± S.E.M (Standard error of measurement)

μmol min^{-1} g^{-1} wet tissue, and average HAD activity was 78.9 ± 3.6 μmol min^{-1} g^{-1} wet tissue (Table 1). The CS:HAD ratio of the cardiac tissue was approximately 0.25 (Table 2.1). An average LDH activity of 239.5 ± 8.9 μmol min^{-1} g^{-1} wet tissue was present in these emu populations (Table 2.1).

There were no significant differences in myoglobin concentration (Fig. 2.4A), CS activity (Fig. 2.4B), or HAD activity (Fig. 2.4C) in cardiac tissue between the two emu populations. The activity of LDH was significantly higher in the Laporte population compared to the Livermore population (Fig. 2.4D). There were no trends in the data that suggested emus with splayed-leg

disorder have a different myoglobin concentration or enzymatic makeup of the cardiac tissue compared to non splayed-leg emus. All results for the myoglobin and enzymatic assays for the control Weddell seal skeletal muscle samples were within the range established from previous studies (Kanatous et al., 2002; Kanatous et al., 2008).

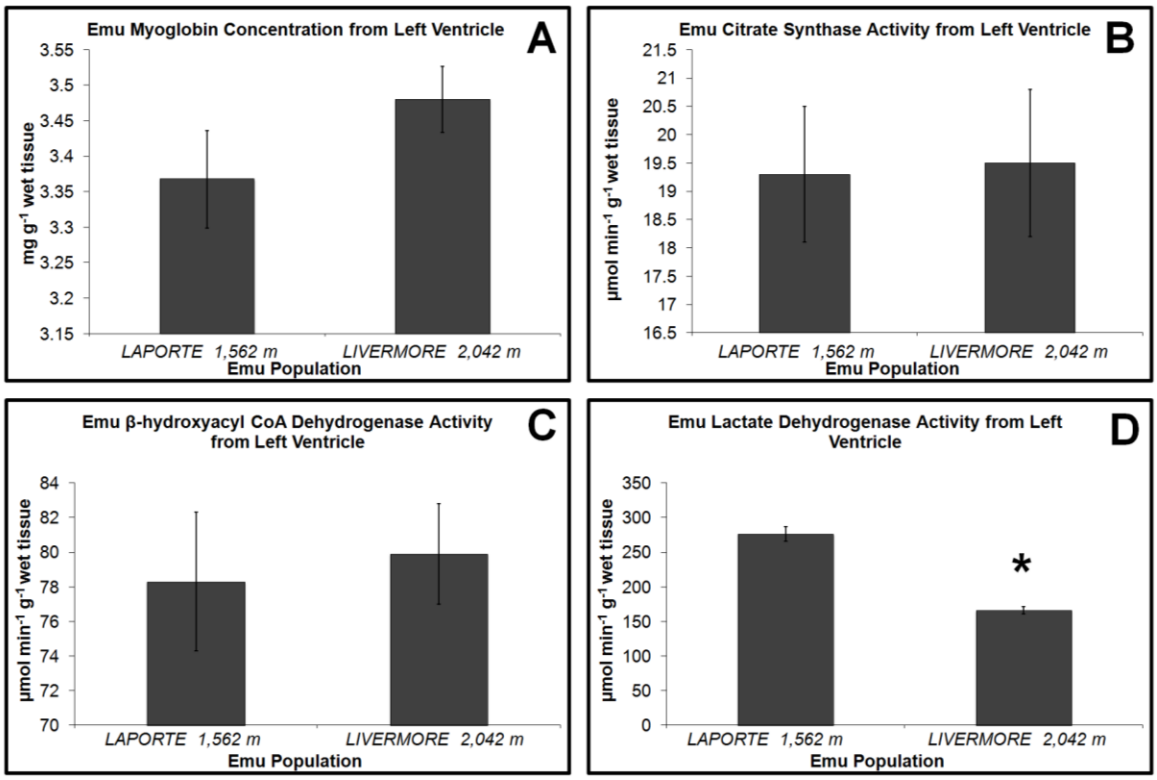

Fig. 2.4. Myoglobin concentration (A), and enzymatic activities of citrate synthase (CS) (B), β-hydroxyacyl CoA dehydrogenase (HAD) (C), and lactate dehydrogenase (LDH) (D) of the left ventricle from the lower elevation (Laporte, 1,562 m, n = 4) and higher elevation (Livermore, 2,042 m, n=8) emu populations. Myoglobin was measured in mg g^{-1} wet tissue and all enzymatic assays were measured in µmol min^{-1} g^{-1} wet tissue. All samples were run as triplicates, and each assay was repeated three times. Significant differences between the two populations are denoted by *****. $P \leq 0.05$; means ± S.E.M (Standard error of measurement).

Discussion

This study was the first to present myoglobin concentration and enzymatic activities from emu cardiac tissue, and the metabolic profile from these Colorado populations provides an estimate of potential oxygen storage and exercise capacity. The high levels of aerobic proteins in

emu cardiac tissue recognized in this study (Table 2.1) supports the hypothesis that even domesticated emus are, physiologically, elite athletes.

Table 2.2. Metabolic Profile of Emu Cardiac Tissue: Comparisons between Vertebrates. Myoglobin concentrations are presented as mg g^{-1} wet tissue; enzyme activities (CS=citrate synthase; HAD= β-hydroxyacyl CoA dehydrogenase; LDH=lactate dehydrogenase) are presented as μmol min^{-1} g^{-1} wet tissue.

Organism	Cardiac Myoglobin	Cardiac CS	Cardiac HAD	Cardiac CS:HAD	Cardiac LDH
Birds					
Emu (domesticated)	**3.41 ± 0.06[a]**	**19.4 ± 1.2[a]**	**78.9 ± 3.6[a]**	**0.25[a]**	**239.5 ± 8.9[a]**
Chicken (domesticated)	2.8[b]	51.0[c]	--	--	1140.0[d]
Little penguin	11.0[e]	--	--	--	--
Pigeon guillemot	16.1[f]	--	--	--	60.0[f]
Pigeon	3.1[g]	127.0[c]	--	--	181.3[h]
Bar-headed goose	6.0[i]	--	--	--	--
House sparrow	--	120.0[c]	--	--	--
Tufted duck	7.0[j]	108.0[j]	23.1[j]	4.7[j]	334.0[j]
Mammals					
Human	--	16.7[k]	--	--	--
Dog (domesticated)	1.8[l]	98.6[l]	24.9[l]	4.0[l]	646.0[l]
Rat	1.7[l]	193.3[l]	27.7[l]	7.0[l]	1347.0[l]

[a] **Green et al. (present study); means ± S.E.M (Standard error of measurement)** -- indicates data not available.
[b] Nishida (1976).
[c] Alp et al. (1976).
[d] Heinova et al. (1999).
[e] Mill and Baldwin (1983).
[f] Haggblom et al. (1988).
[g] Pages and Planas (1983).
[h] Lumeij et al. (1988).
[i] Saunders and Fedde (1991)
[j] Turner and Butler (1988).
[k] Veerkamp et al. (1985).
[l] Polasek et al. (2006).

Emus also appear to have a similar metabolic adaptation to that of flying birds, in using high levels of fat oxidation in cardiac tissue to accomplish strenuous aerobic activity (Table 2.2).

The average cardiac myoglobin concentration from the Livermore and Laporte emus was 3.41 ± 0.06 mg g^{-1} wet tissue, which is higher than that of a domesticated chicken (*Gallus gallus domesticus*), 2.8 mg g^{-1} wet tissue (Nishida, 1976) (Table 2.1), but much lower than the levels of myoglobin seen in the cardiac tissue of diving birds like the little penguin (*Eudyptula minor*) and pigeon guillemot (*Cepphus columba*), 11.0 mg g^{-1} wet tissue (Mill and Baldwin, 1983) and 16.1 mg g^{-1} wet tissue (Haggblom et al., 1988) respectively (Table 2.1). Diving birds store oxygen in their bodies *via* myoglobin in skeletal and cardiac tissue in order to undergo long, breath-hold

dives, while relying on their oxidative metabolism. Flying birds also use myoglobin to store oxygen, though they have the ability to ventilate while exercising, and thus are less reliant on endogenous stores of oxygen, and generally have less myoglobin in cardiac tissue compared to diving birds (Saunders and Fedde, 1994). Captive emus have myoglobin levels that are more similar to those of the strong-flying pigeon (*Columba livia*), 3.1 mg g^{-1} (Pages and Planas, 1983) and high-flying bar-headed goose (*Anser indicus*), 6.0 mg g^{-1} (Saunders and Fedde, 1991) (Table 2.1). Even though emus in North America are captive ground birds, they still have similar myoglobin levels to athletic flying birds. These relatively high myoglobin levels in heart and skeletal muscle tissue (Patak and Baldwin, 1993) suggest emus rely on myoglobin to enhance their endurance capabilities. The myoglobin in emu heart tissue, and in cardiac tissue of other animals, binds oxygen so the tissue has a nearby oxygen supply to supplement the tissue during long-duration exercise (Wittenberg et al., 1975). These emu populations were born and raised at high elevation, and the relatively high concentrations of myoglobin may also be an adaptation to living at an elevation with a lower partial pressure of oxygen.

Cardiac CS levels for these farm-raised emus are lower than the activity seen in birds with a smaller mass (Saunders and Fedde, 1994), like the house sparrow (*Passer domesticus*), which displays a cardiac CS activity of 120.0 µmol min^{-1} g^{-1} wet tissue (Alp et al., 1976) (Table 2.1). Emus have a large body mass, and therefore a lower mass-specific metabolism, so these results are expected. However, the emus from the study had a higher activity of CS compared to an animal of a similar average body mass, an adult human (*Homo sapiens*), which has a CS activity in cardiac tissue of approximately 16.7 µmol min^{-1} g^{-1} wet tissue (Veerkamp et al., 1985) (Table 2.1). Although CS cardiac values are rarely collected from healthy humans, skeletal muscle CS activity from human biopsies also tends to be lower than that in skeletal muscle of

emus. For example, untrained humans have an average CS activity of 12.3 μmol min^{-1} g^{-1} wet tissue in pelvic limb skeletal muscle (Leek et al., 2001), while domesticated emus have an average CS activity of 16.3 μmol min^{-1} g^{-1} wet tissue (T. L. Green, unpublished results). The exercised pelvic limb skeletal muscle of a trained human has a CS activity of 21.8 μmol min^{-1} g^{-1} wet tissue (Leek et al., 2001) while pelvic limb muscle of wild Australian emus has an average CS activity of 72.3 μmol min^{-1} g^{-1} wet tissue (Patak and Baldwin, 1993). Normally, mammals tend to have a higher activity of CS in their cardiac tissue compared to birds of similar mass. For example, a house sparrow has a cardiac CS activity of about 120.0 μmol min^{-1} g^{-1} wet tissue while a laboratory mouse of similar mass has a cardiac CS activity of about 146.0 μmol min^{-1} g^{-1} wet tissue (Alp et al., 1976) (Table 2.1). For unknown reasons, birds appear to have lower levels of CS in their cardiac tissue, but are equally athletic as mammals of comparable size. This supports the view of emus having high aerobic potential because when compared to mammals of similar mass, emus are the organism with the higher CS activity.

HAD activity is not often analyzed in cardiac tissue, especially in birds. However, in emus, HAD activity is much higher than in the cardiac tissue of the tufted duck (*Aythya fuligula*), 23.1 μmol min^{-1} g^{-1} wet tissue (Turner and Butler, 1988), dog (*Canis familiaris*), 24.9 μmol min^{-1} g^{-1} wet tissue, and rat (*Rattus norvegicus*), 27.7 μmol min^{-1} g^{-1} wet tissue (Polasek et al., 2006) (Table 2.1). This is surprising, because emus have a larger body mass compared to both of these animals. This illustrates these domesticated emus had relatively high levels of β-oxidation in cardiac tissue compared to dogs and rats. The ratio of CS:HAD activity gives an estimate of how reliant an animal is on lipids for metabolic fuel. In dogs this ratio is approximately 4, and in rats it is 7 (Polasek et al., 2006) (Table 2.1). A ratio of 1 indicates the cardiac system is 100% reliant on fat as a fuel source, and the higher the ratio, the less reliant an animal is on fat. Emus have a

CS:HAD ratio of approximately 0.25 in their cardiac tissue (Table 2.1), indicating emus have the capacity to rely completely on fat oxidation for a cardiac fuel source. In order to fuel long-distance flights, birds are thought to rely heavily upon fat utilization during migrations (Blem, 1980; Dingle, 1996; Guglielmo, 2010). It has been found that white-throated sparrows (*Zonotrichia albicollis*) actually upregulate fatty acid transporters in the *M. pectoralis major* (primary flight muscle) during the migratory season. It is likely an evolutionary adaptation for flighted birds to rely on lipid storage and usage for constant exercise during migration, because fat is a relatively light and energy-dense fuel source (Jenni and Jenni-Eiermann, 1998). Flighted birds cannot afford to put on much extra mass, or it could hinder the migration process (Jenni and Jenni-Eiermann, 1998). Although domesticated emus are generally fed a diet that only includes 5.0% crude fat, it appears that they preferentially use fat as a metabolic fuel during exercise (or during cold winter months) in a similar way to flying migrating birds. Although the low CS:HAD ratio in emus is fairly rare for muscle tissue, a comparable ratio has been identified in Weddell seal skeletal muscle, which is about 0.3 (Kanatous et al., 2002). Weddell seals are Antarctic diving mammals that consume lipid-rich prey, and in turn, store lipids in a subcutaneous fat layer which is used to fuel aerobic metabolism for long foraging dives (Kooyman and Ponganis, 1998; Burns, 1999; Kanatous et al., 2002). Because emus from this study were consuming ratite pellets with a relatively low crude fat content, emus must be metabolically managing their lipids differently than seals. Due to a low CS:HAD ratio and high levels of myoglobin in skeletal muscle tissue, Weddell seals have the capacity for high endurance capabilities (Kanatous et al., 2002). These farm-raised emus have a similar CS:HAD ratio to Weddell seals, and may therefore rely on a similar mechanism in the cardiac tissue to support endurance exercise under low-oxygen situations.

The average LDH level of the two populations was 239.5 ± 8.9 μmol min^{-1} g^{-1} wet tissue (Table 2.1). This again reflects the difference in enzymatic activity in relation to body mass. Tufted ducks have higher LDH activity, about 334.0 μmol min^{-1} g^{-1} wet tissue (Turner and Butler, 1988), compared to the emus from this study, but a lower body mass (Table 2.2). Highly active birds like tufted ducks and pigeon guillemots (Haggblom et al., 1988) have much lower LDH activity in cardiac tissue compared to more sedentary birds like the domesticated chicken; 1140.0 μmol min^{-1} g^{-1} wet tissue (Heinova et al., 1999) (Table 2.2). It is possible that more active birds have higher levels of aerobic proteins, and lower levels of anaerobic proteins. Because many birds, including the tufted duck and pigeon guillemot, are elite endurance athletes, an inherent predisposition for high levels of aerobic proteins support this type of activity. As seen with living emus, they have effective anaerobic ability (O'Brien, 1990), though the even higher aerobic capacity equips emus to be even more impressive endurance athletes.

These assay data indicate that emus have high levels of metabolic proteins in their heart tissue to support elite activity. In regard to cardiac tissue, farm-raised emus do not resemble other domesticated birds like the chicken. Instead, their hearts are biochemically structured more similarly to those of flighted migrating birds. Emus are impressive athletes that are inherently predisposed for fat-burning, aerobic runs, like many other terrestrial prey species.

Perspectives

By determining the metabolic profile of emu muscle tissue, researchers can begin to understand how metabolic processes are regulated in emus. This study establishes a baseline for healthy emu cardiac tissue, and allows for a number of potential projects to be conducted in the future.

Factors, such as age and body mass, can affect metabolism greatly. It is unknown whether large elevation variations can cause metabolic cardiac differences in emus. Elevation differences have been shown to have an effect on other vertebrate groups. In previous studies with rat myocardium, it was determined that there is a significant change in myoglobin concentration, CS activity, and LDH activity due to simulated elevation differences (Esteva et al., 2009). Hypoxia (low-oxygen environment) has been shown to have an effect on cardiac processes in emu hatchlings (Shah et al., 2010). Although heart rate was unchanged, there was a significant difference in β-adrenergic tone between emu hatchlings in normoxic and hypoxic environments (Shah et al., 2010). No major differences were observed in metabolic protein activity between the two ranches, despite slight elevation differences. Future emu cardiac research should focus upon birds raised at sea level to see if significant differences exist between them (a baseline population) and high elevation populations. Though domesticated emus are inherently predisposed for relatively high levels of aerobic proteins, it is also possible that wild emus (because they are thought to be more active than domesticated emus) would have even higher myoglobin content and greater metabolic enzyme activities. It is essential to further analyze tissue from wild emus in future studies to understand the effects that domestication and limited exercise have upon avian physiology.

Investigation of the metabolic profile of skeletal muscle tissue of domesticated emus could be expanded to make comparisons between emus under varying climate conditions, diets, activity levels, ages, and disease states. Because hind limb orthopedic disorders are fairly common in domesticated ratites on farms and in zoos (Gnad et al., 1996; Minnaar, 1998; Rothschild and Rühli, 2007; Cooper et al., 2008), these defects have been of interest to emu caretakers. A specific splayed-leg disorder is especially prevalent in farm-raised emus, and is a

growing concern. The cause of splayed-leg disorder in ratites is thought to be multifactorial (Gnad et al., 1996); genetics, nutrition, husbandry, and injuries are thought to be possible causes for this pathology (Gnad et al., 1996; Jenkins, 1996). The etiology of this disorder is unknown. It is not understood whether skeletal defects cause muscle atrophy, or if muscular dysfunctions alter bone formation. Investigating the metabolic properties of skeletal muscle tissue from captive emus will set a baseline for what healthy emus look like, and will allow comparisons to be made between these animals, and those affected by splayed-leg disorder.

Additionally, the evolutionary history and anatomy of emus make them excellent models for branching into comparative studies with theropod dinosaurs (Padian and Olsen, 1989; Castanet et al., 2000; Schweitzer et al., 2005; Milàn, 2006; Breithaupt et al., 2007). Emus exhibit a fast period of growth after hatching from eggs (Minnaar, 1998; Breithaupt et al., 2007). The combination between this rapid development, skeletal similarities, and the parental care exhibited by adult male emus (Minnaar, 1998), make them excellent modern analogs for paleontologists attempting to reconstruct theropod dinosaur development, growth, and behavior.

Conclusions

Here, the metabolic profile of ratite cardiac tissue was classified for the first time. Due to high levels of aerobic proteins, it can be concluded that even domesticated emus are predisposed to be elite avian athletes. The data illustrate that emu heart tissue is inherently supported by myoglobin and metabolic enzymes for these highly athletic birds during exercise. Domesticated emu cardiac tissue has similar metabolic characteristics to active, flighted birds (pigeons and geese) rather than farm-raised, sedentary birds (chickens). Emus, and active birds that rely on aerobic metabolism have a number of similarities, including high levels of aerobic proteins

(myoglobin, citrate synthase, and β-hydroxyacyl CoA dehydrogenase) in cardiac tissue that are fueled by β-oxidation. This suggests that, although behaviorally emus are both efficient sprinters and long-distance runners, this species relies more readily upon aerobic metabolism to fuel activity. Emu ranching in the United States allows a unique opportunity to study the physiology of these large, terrestrial birds. The knowledge gained from this study can be used to better understand metabolism and ontogeny of domesticated emus. This study begins to set the physiological groundwork by identifying a metabolic profile for farm-raised emu cardiac tissue.

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CHAPTER 3

Striding for excellence: Determination of the metabolic profile of specific skeletal muscles from normally developing and splayed-leg emus (*Dromaius novaehollandiae*) from Livermore, Colorado

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Introduction

Paleognathous birds are the small lineage that includes tinamous (*Tinamus*, *Nothocercus*, *Crypturellus*, *Rhynchotus*, *Nothoprocta*, *Nothura*, *Taoniscus*, *Eudromia*, and *Tinamotis*), rheas (*Rhea*), kiwis (*Apteryx*), ostriches (*Struthio*), emus (*Dromaius*), and cassowaries (*Casuarius*). Ratites are specifically the paleognathous birds that are fully flightless (all above except tinamous). Emus (*Dromaius novaehollandiae* Latham 1790) are a ratite species native to Australia, though they were imported to North America, and later raised as livestock animals for oil, meat, leather, eggs, and feathers. Over one million emus are currently raised throughout the United States for commercial products (American Emu Association, 2009; Green et al., *in review*).

Emus are a unique model organism for physiological studies because they are exceptional athletes. They can sprint up to 50 km hr^{-1} (O'Brien, 1990) and can run for long distances without stopping. The hindlimb anatomies of emus (Patak and Baldwin, 1993), ostriches (Smith et al., 2006), and rheas (Piasso, 2010) have been classified to aid in the understanding of ratite biomechanics. Some previous studies have investigated skeletal muscle physiology of wild Australian emus, though domesticated emu tissue has not been extensively studied. The metabolic profile and fiber typing of a variety of pelvic limb skeletal muscles were determined from emus from Western Victoria, Australia in 1993 (Patak and Baldwin, 1993). The data

collected from these techniques suggested that emu pelvic limb skeletal muscles were similar in metabolic makeup (highly aerobic) and comparative in mass to pectoral muscles of flighted birds (25% in emus; 10–35% in flighted birds) (Hartman, 1961; Patak, 1988; Patak and Baldwin, 1993). This reliance on aerobic metabolism is also demonstrated by the metabolic profile of domesticated emu cardiac tissue, which is more similar to that of flying migratory birds (Green et al., *in review*).

The magnitude of aerobic reliance is dependent on whether an animal is inherently predisposed for sprinting (anaerobic) or endurance (aerobic) activity. For example, predatory animals may depend more upon anaerobic metabolism in order to fuel fast bursts of speed, while the muscles of prey animals may be fueled more by aerobic metabolism (Lindstedt, 1991; Williams et al., 1997). Although this predator-prey model is a wonderful example, the amount of aerobic and anaerobic metabolism an animal relies on during exercise may be dependent on numerous factors. An animal's environment (Kooyman and Ponganis, 1998; Kanatous et al., 1999) and body mass (Jones and Lindstedt, 1993) can play a major role in influencing the metabolic profile. Animals can accomplish elite athletic performance regardless of which metabolic system they use more for activity. The African cheetah (*Acinonyx jubatus*), a fast terrestrial runner that can reach speeds of up to 103 km hr^{-1} (Sharp, 1997), relies mostly on anaerobic metabolism for sprinting (Williams et al., 1997). In contrast, pronghorn (*Antilocapra americana*) can reach alleged speeds of 100 km hr^{-1} while relying on aerobic metabolism for endurance running (Lindstedt, 1991). This study will investigate muscle proteins in domesticated emus to determine whether specific pelvic limb skeletal muscles are more inherently suited for anaerobic or aerobic activity. The metabolic profile of domesticated emu skeletal muscle had never been identified, and it is necessary for determining a baseline for

healthy animals. The baseline metabolic profile will also allow for comparative studies between healthy and pathologic domesticated emus. Hindlimb orthopedic disorders (especially trauma-related) are fairly common in domesticated ratites on farms and in zoos (Gnad et al., 1996; Minnaar, 1998; Rothschild and Rühli, 2007; Cooper et al., 2008), and these disorders have been of interest to emu caretakers. A specific condition called splayed-leg disorder is especially prevalent in farm-raised emus, and is a growing concern (Fig. 3.1). The cause of splayed-leg disorder is thought to be multifactorial (Gnad et al., 1996); low genetic diversity, poor nutrition, lack of exercise, and injuries are thought to be possible causes for ratite splayed-leg disorder (Gnad et al., 1996; Jenkins, 1996). Although studies have identified osteologic disorders in ratites (Rothschild and Rühli, 2007), it is unknown whether defective bones cause muscles to become weak, or if defective muscles cause bones to twist (Fig. 3.1). In this study the metabolic properties were measured in muscle tissue from a splayed-leg individual to determine if differences exist between it and its healthy counterparts.

Analyzing the metabolic properties of skeletal muscle tissue from captive emus sets a physiological baseline, and specific pelvic limb muscles were also analyzed to see whether specific muscles rely more on aerobic or anaerobic metabolism. It was predicted that domesticated emu skeletal muscles will generally be more aerobic. A baseline metabolic profile also allowed comparisons to be made between these animals, and ones with orthopedic disorders. It was unclear whether splayed-leg birds would possess skeletal muscle atrophies. If there are atrophies in the three analyzed muscles, it is expected that splayed and non-splayed limbs will have lower activities of metabolic enzymes and myoglobin concentrations than the normally developing limbs.

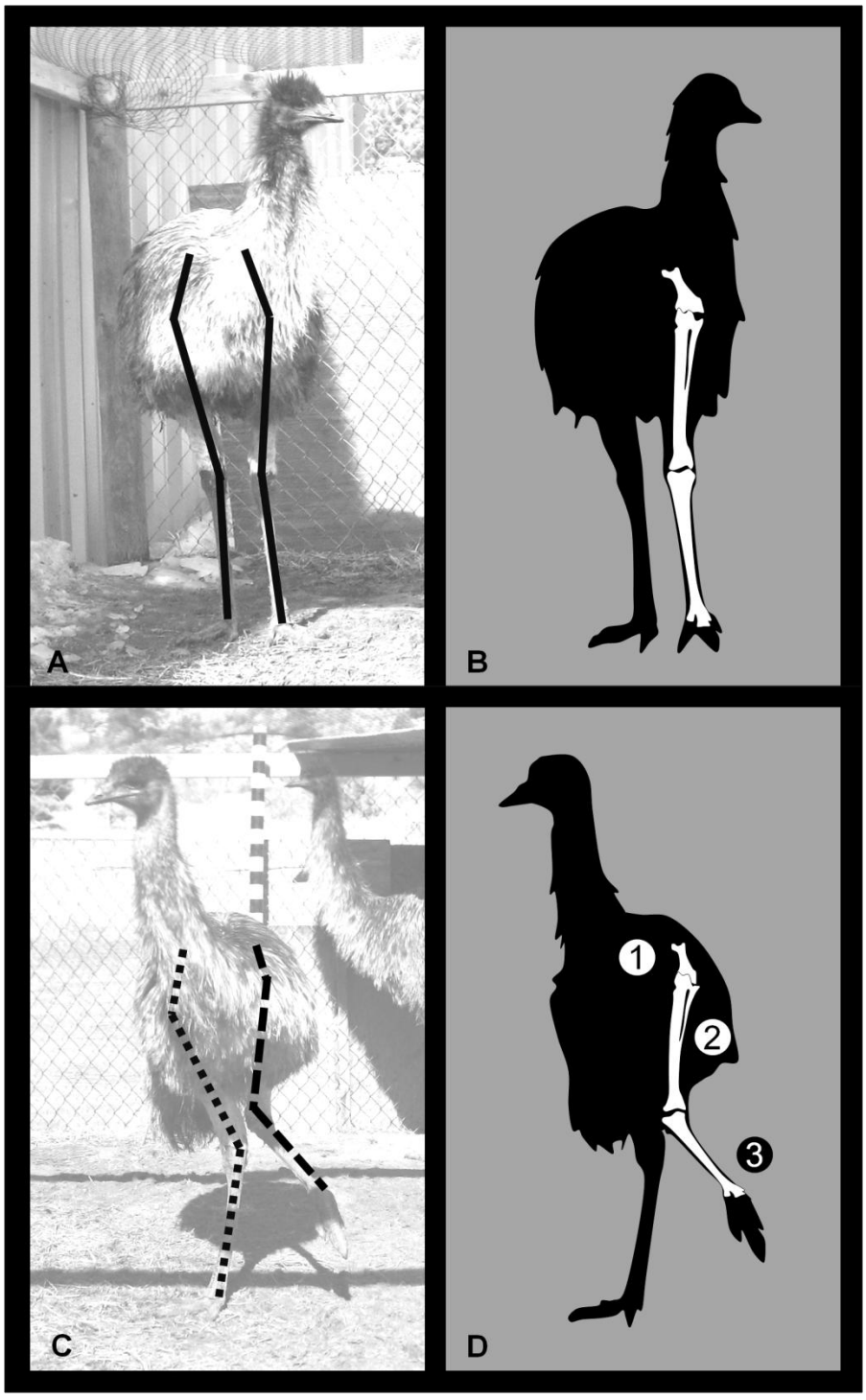

Fig. 3.1. (A) A normally-developing sub-adult emu. The solid lines illustrate how both limbs are oriented directly below the emu's body. (B) The hindlimb bones (femur, tibiotarsus, fibula, and tarsometatarsus) are untwisted in a normally-developing emu. (C) A sub-adult emu affected by splayed-leg disorder. The dotted line represents the non-splayed limb, and the dashed line represents the splayed limb. Splayed-leg disorder causes one limb to point laterally out from the body, making it difficult for the animal to walk properly. (D) Though the femur (1) is affected minimally by the splayed-leg pathology, the tibiotarsus (including fibula) (2) and tarsometatarsus (3) both become twisted. (Illustrations and photos by Todd L. Green).

Materials and methods

Study Animals

Emus from this study (n=4) were raised in an outdoor pen at an elevation of 2,042 m (6,700 ft) at Rabbit Creek Emu Ranch, in Livermore, Colorado, USA. Of the four emus, three were sub-adults (between 1-2 years old) and one was a breeding adult female (> 4 years old). The age classifications used for this study were based upon physical and behavioral indications of sexual maturity and breeding status of the emus (T. L. Green, unpublished). One of the sub-adult birds possessed splayed-leg disorder. Three limb states were designated for the analysis: a control limb (from emus without any sign of limb disorder), a non-splayed limb (supportive limb from a splayed-leg bird), and a splayed limb (splayed limb from a bird with splayed-leg disorder) (Fig. 3.1). Emus were butchered by workers at Wind River Processing Inc. (Thermopolis, WY, USA) on March 15, 2010. After the emus were butchered, the limbs were placed in an industrial cooler for one hour, before skeletal muscle samples were collected from the mid-belly of three different hindlimb muscles: *M. gastrocnemius medialis* (GM - inside drum), *M. iliofibularis* (IFB - fan fillet), and *M. iliofemoralis externus* (IFME - oyster fillet) (Fig. 3.2). The small skeletal muscle samples were then immediately wrapped in aluminum foil and placed in a charged liquid nitrogen dewar, until they could be stored in a -80°C freezer at Colorado State University for later analysis of metabolic characteristics. Adult Weddell seal (*Leptonychotes weddellii*) skeletal muscle samples, originally collected in October 2006 by S. B. Kanatous McMurdo Sound, Antarctica, were used as an internal control for all assays. These samples were readily available, and have been tested in a number of studies in our Colorado laboratory.

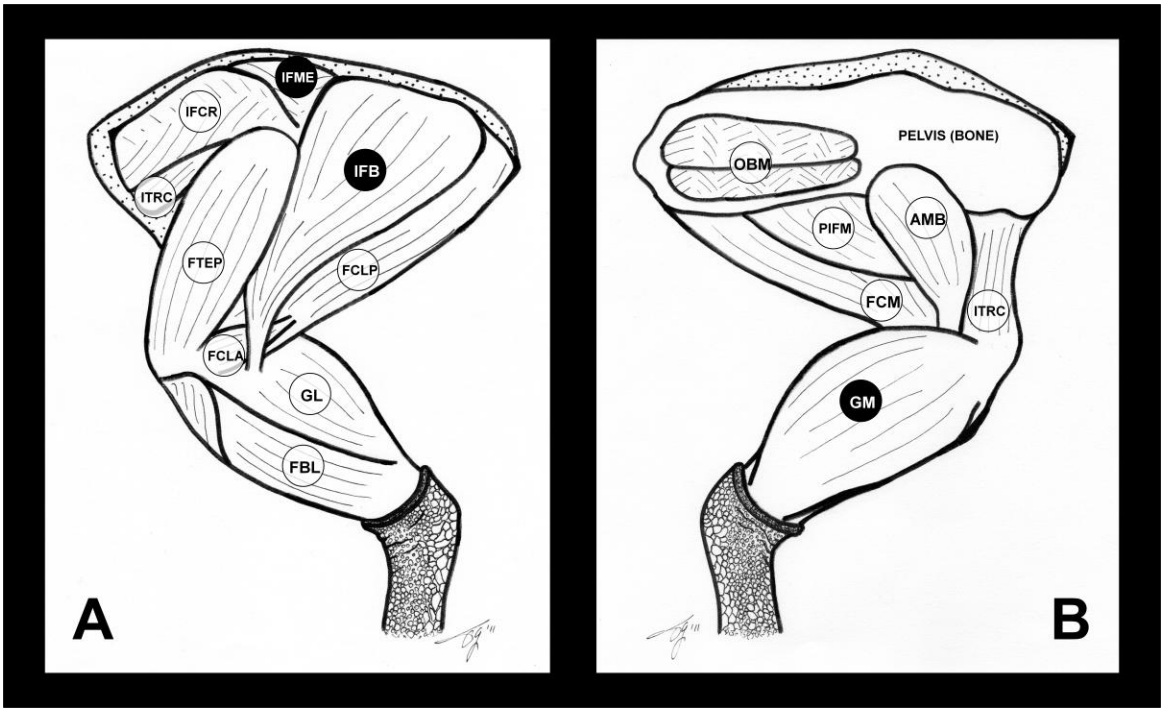

Fig. 3.2. (A) The lateral view of a left emu pelvic limb. *M. gastrocnemius lateralis* (GL); *M. fibularis longus* (FBL); *M. femorotibialis externus p. proximalis* (FTEP); *M. iliotrochantericus cranialis* (ITRC); *M. iliofemoralis cranialis* (IFCR); *M. flexor cruris lateralis p. pelvic* (FCLP); *M. flexor cruris lateralis p. accessoria* (FCLA). Some of the more superficial muscles were removed in order to view *M. iliofibularis* (IFB - fan fillet) and *M. iliofemoralis externus* (IFME - oyster fillet), the two upper pelvic limb muscle analyzed for the study. (B) The medial view of a left emu pelvic limb. *M. obturatorius medialis* (OBM); *M. ambiens* (AMB); *M. flexor cruris medialis* (FCM); *M. iliotrochantericus cranialis* (ITRC); *M. puboishiofemoralis medialis* (PIFM). The white area on the limb is the pelvis bone. The lower limb muscle analyzed in this study was *M. gastrocnemius medialis* (GM - inside drum). Dark circles=muscles analyzed in the current study; light circles=other emu pelvic limb muscles not analyzed in the current study. (Redrawn by Todd L. Green, adapted from Patak and Baldwin, 1998)

Myoglobin Assays

The concentration of myoglobin, an oxygen-binding heme protein, was determined for the skeletal muscle tissue. For all myoglobin and enzyme assays 250 mg of skeletal muscle was homogenized with a hand glass homogenizer. A homogenization buffer was made with 79% PBS (phosphate buffered saline), 1% Tween-20, 20% glycerol, 0.001M DTT (dithiothreitol), and a protease inhibitor tablet (1/10 µl). Tissue was weighed and homogenized (0°C), and the original dilution recorded. The resulting homogenates were clarified in a centrifuge at 10,000 x ***g*** for 10 min. The supernatant was collected, aliquoted, and stored in a -80°C freezer until it was used for the assays. Protein concentrations were determined for all emu skeletal muscle samples

by using Pierce Coomassie Plus Protein Assay Reagent (Pierce Chemicals, Rockford, IL, USA) before enzymatic assays were performed. Myoglobin assays were performed in accordance with the Reynafarje (1963) method adapted by Kanatous et al., 2002 with the following modifications: a portion (100 µl) of the homogenate was further diluted with 650 µl of phosphate buffer (0.04 mol l^{-1}, pH 6.6). The resulting mixture was centrifuged for 50 min at 28,000 x ***g*** at 4°C. The supernatant was bubbled with 99.9% carbon monoxide for 3 min. Spectrophotometric absorbance was measured at 538 nm and 568 nm, and the concentration of myoglobin in mg g^{-1} wet mass of muscle was calculated as: (Abs_{538}–Abs_{568}) X 5.865 [(1.5/0.5) X (dilution of sample)].

Enzyme Assays

Enzymatic activity of citrate synthase (CS–aerobic potential), beta-hydroxyacyl CoA dehydrogenase (HAD–aerobic, β-oxidation), and lactate dehydrogenase (LDH–anaerobic potential) were determined for the skeletal muscle tissue samples. The assay conditions were set according to previous studies (Reed et al., 1994; Kanatous et al., 2002): *Citrate synthase* (CS; EC 4.1.3.7): 50 mmol l^{-1} imidazole; 0.25 mmol l^{-1} 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB); 0.4 $mmol^{-1}$ acetyl CoA; and 0.5 mmol l^{-1} oxaloacetate, pH 7.5 at 37°C; ΔA_{412}, ε_{412}=13.6. *β-hydroxyacyl CoA dehydrogenase* (HAD; EC 1.1.1.35): 50 mmol l^{-1} imidazole; 1 mmol l^{-1} EDTA; 0.1 mmol l^{-1} acetoacetyl CoA; and 0.15 mmol l^{-1} NADH, pH 7.0 at 37°C; ΔA_{340}, ε_{340}=6.22. *Lactate dehydrogenase* (LDH; EC 1.1.1.27): 50 mmol l^{-1} imidazole; 0.15 mmol l^{-1} NADH, pH 7.0 at 37°C; and 1 mmol l^{-1} pyruvate; ΔA_{340}, millimolar extinction coefficient ε_{340}=6.22. Myoglobin and enzyme assays were determined by using a BioTek Synergy HT Multi-detection microplate reader (Winooski, VT, USA). Specific enzyme activities (µmol min^{-}

^{1} g^{-1} wet mass of muscle) were calculated from the rate of change of the assay absorbance at the maximal linear slope and normalized to wet tissue weight. The CS:HAD ratio was calculated in order to estimate the dependence of aerobic metabolism upon lipid oxidation.

Statistical Analyses

All muscle samples were run as triplicates, and each assay was repeated three times for the determination of skeletal muscle metabolic profile (Table 3.1). Skeletal muscle samples from an adult Weddell seal were used as controls for the assays. The statistical analyses were done *via* SigmaStat 2.0 (Ashburn, VA, USA). Analysis of variance (one-way ANOVA) with Tukey *post-hoc* tests were used ($P \leq 0.05$), and the results given as means ± S.E.M.

Table 3.1. Metabolic Profile of Specific Domesticated Emu Skeletal Muscles

Emu Pelvic Limb Muscle	Healthy Limb (Control)	Non-splayed Limb (Splayed-leg emu)	Splayed Limb (Splayed-leg emu)
M. gastrocenemius medialis (GM)			
Mb	2.74 ± 0.03	5.16 ± 0.04 ***†**	3.34 ± 0.04 *****
CS	12.14 ± 1.21	18.27 ± 0.31 ***†**	11.37 ± 0.41
HAD	14.05 ± 0.52	32.79 ± 1.26 ***†**	16.25 ± 1.06
CS:HAD	0.88	0.55	0.70
LDH	905.44 ± 67.58	1254.15 ± 103.10	1451.45 ± 142.37 *****
M. iliofibularis (IFB)			
Mb	3.04 ± 0.08	4.15 ± 0.06 ***†**	2.80 ± 0.09
CS	18.43 ± 0.85 [a]	16.52 ± 1.36	16.32 ± 0.98
HAD	23.04 ± 2.28 [a]	28.49 ± 3.29	19.02 ± 0.98
CS:HAD	0.80	0.58	0.86
LDH	913.08 ± 57.92	1029.32 ± 28.54	1165.44 ± 39.40 *****
M. iliofemoralis externus (IFME)			
Mb	4.43 ± 0.07 [ab]	6.51 ± 0.08 ***†**	5.87 ± 0.18 *****
CS	18.23 ± 0.40 [a]	25.26 ± 0.69 ***†**	22.73 ± 0.71 *****
HAD	28.66 ± 0.91 [ab]	45.69 ± 2.45 ***†**	35.85 ± 1.26 *****
CS:HAD	0.64	0.55	0.63
LDH	947.65 ± 37.70	1260.27 ± 36.14 *****	1307.68 ± 50.73 *****

Skeletal muscle metabolic profile from domesticated emus raised at Rabbit Creek Emu Ranch in Livermore, Colorado. Myoglobin (Mb) concentrations are presented as mg g^{-1} wet tissue; enzyme activities (CS=citrate synthase; HAD= β-hydroxyacyl CoA dehydrogenase; LDH=lactate dehydrogenase) are presented as µmol min^{-1} g^{-1} wet tissue. Means ± S.E.M (Standard error of measurement)

[a] Significantly higher than GM
[b] Significantly higher than IFB

***** Significantly higher than control limb
† Significantly higher than splayed limb

Results

Myoglobin

The myoglobin concentrations in the control (healthy) emu limb muscles were 2.74 ± 0.03 mg g^{-1} wet tissue in the *M. gastrocnemius medialis* (GM), 3.04 ± 0.08 mg g^{-1} wet tissue in the *M. iliofibularis* (IFB), and 4.43 ± 0.07 mg g^{-1} wet tissue *M. iliofemoralis externus* (IFME) (Table 3.1). For the non-splayed limbs from a splayed-leg bird the myoglobin levels were 5.16 ± 0.04 mg g^{-1} wet tissue in the GM, 4.15 ± 0.06 mg g^{-1} wet tissue in the IFB, and 6.15 ± 0.08 mg g^{-1} wet tissue IFME (Table 3.1). The muscles in the splayed limb had myoglobin concentrations of 3.34 ± 0.04 mg g^{-1} wet tissue in the GM, 2.80 ± 0.09 mg g^{-1} wet tissue in the IFB, and 5.87 ± 0.18 mg g^{-1} wet tissue IFME (Table 3.1). In all three pelvic limb muscles, there was a significantly greater myoglobin concentration in the non-splayed limb compared to the control and splayed limb (Fig. 3.3). For the GM and IFME, the splayed limb also had greater concentrations of myoglobin relative to the control limb (Fig. 3.3). The Weddell seal skeletal muscle controls were within the range of previously reported values (Kanatous et al., 2002; Kanatous et al., 2008).

Enzyme activities

The citrate synthase (CS) activities in the control limb were 12.14 ± 1.21 μmol min^{-1} g^{-1} wet tissue in the GM, 18.43 ± 0.85 μmol min^{-1} g^{-1} wet tissue in the IFB, and 18.23± 0.40 μmol min^{-1} g^{-1} wet tissue in the IFME (Table 3.1). For the non-splayed limb, CS activities were 18.27 ± 0.31 μmol min^{-1} g^{-1} wet tissue in the GM, 16.52 ± 1.36 μmol min^{-1} g^{-1} wet tissue in the IFB, and 25.26 ± 0.69 μmol min^{-1} g^{-1} wet tissue in the IFME (Table 3.1). The CS activities in the splayed limb were 11.37 ± 0.41 μmol min^{-1} g^{-1} wet tissue in the GM, 16.32 ± 0.98 μmol min^{-1} g^{-1}

wet tissue in the IFB, and 22.73 ± 0.71 μmol min^{-1} g^{-1} wet tissue (Table 3.1). The CS activities in the GM and IFME were significantly higher in the non-splayed limb compared to the control limb (Fig. 3.3). The CS activities were also significantly higher in the GM and IFME of the non-splayed limb relative to the splayed limb (Fig. 3.3). The IFME was the only muscle of the splayed limb that was significantly higher in CS activity compared to the control limb (Fig. 3.3). There were no differences in citrate synthase activity in the IFB between the three limb states (Fig 3.3).

The β-hydroxyacyl CoA dehydrogenase (HAD) activity in healthy emu limbs were 14.05 ± 0.52 μmol min^{-1} g^{-1} wet tissue in GM, 23.04 ± 2.28 μmol min^{-1} g^{-1} wet tissue in the IFB, and 28.66 ± 0.91 μmol min^{-1} g^{-1} wet tissue in IFME (Table 3.1). The HAD activities in the non-splayed limb were 32.79 ± 1.26 μmol min^{-1} g^{-1} wet tissue in GM, 28.49 ± 3.29 μmol min^{-1} g^{-1} wet tissue in IFB muscle, and 45.69 ± 2.45 μmol min^{-1} g^{-1} wet tissue in the IFME (Table 3.1). For the splayed limb the HAD activities were 16.25 ± 1.06 μmol min^{-1} g^{-1} wet tissue in the GM, 19.02 ± 0.98 μmol min^{-1} g^{-1} wet tissue in the IFB, and 35.85 ± 1.26 μmol min^{-1} g^{-1} wet tissue in the IFME (Table 3.1). The non-splayed limb had significantly greater HAD activity compared to the control limb in both the GM and IFME muscles (Fig. 3.3). The non-splayed limb also had significantly higher HAD activity in the GM and IFME than the splayed limb (Fig. 3.3). The splayed-limb IFME was the only limb muscle that had greater HAD activity compared to the control limb (Fig. 3.3). There were no significant differences in IFB HAD activity between the limb states (Fig. 3.3).

In the healthy limb, the CS:HAD ratios in the GM, IFB, and IFME were respectively 0.88, 0.80, and 0.64 (Table 3.1). For the non-splayed limb the CS:HAD ratios were 0.55 in the

GM, 0.58 in the IFB, and 0.55 in the IFME (Table 3.1). The splayed limb had CS:HAD ratios of 0.70, 0.86, and 0.63 in the GM, IFB, and IFME, respectively (Table 3.1).

The control limb lactate dehydrogenase (LDH) activities were 905.44 ± 67.58 μmol min^{-1} g^{-1} wet tissue in the GM muscle, 913.08 ± 57.92 μmol min^{-1} g^{-1} wet tissue in the IFB, and 947.65 ± 37.70 μmol min^{-1} g^{-1} wet tissue in the IFME (Table 3.1). LDH activities of the non-splayed limb were 1254.15 ± 103.10 μmol min^{-1} g^{-1} wet tissue in the GM, 1029.32 ± 28.54 μmol min^{-1} g^{-1} wet tissue in the IFB, and 1260.27 ± 36.14 μmol min^{-1} g^{-1} wet tissue in the IFME muscle (Table 3.1). The LDH activities of the splayed limb were 1451.45 ± 142.37 μmol min^{-1} g^{-1} wet tissue in the GM, 1165.44 ± 39.40 μmol min^{-1} g^{-1} wet tissue in the IFB, and 1307.68 ± 50.73 μmol min^{-1} g^{-1} wet tissue in the IFME (Table 3.1). The splayed limb had the highest activity of LDH activity in all three muscles (Fig. 3.3). LDH was significantly higher in the splayed limb compared to the control limb in the three pelvic limb muscles (Fig 3.3). The non-splayed limb had significantly higher LDH activity in the IFME compared to the normally developing limb (Fig. 3.3). All enzymatic data from Weddell seal skeletal muscle controls were consistent with previous published results (Kanatous et al., 2002; Kanatous et al., 2008).

Discussion

This study measured the metabolic profile of domesticated emu skeletal muscle for the first time (Table 3.1). All three skeletal muscles had a low CS:HAD ratio (0.88, 0.80, and 0.64), exhibiting their high reliance on β-oxidation to fuel aerobic metabolism (Table 3.1), although the CS:HAD ratio was not as low as previously determined in domesticated emu cardiac tissue (0.25) (Green et al., *in review*). Specific muscles differed in their amount of aerobic reliance, and there was a distinct difference between the more distal GM compared to the more proximal

IFB and IFME, in that the GM had less aerobic potential. Though there are 20% more muscles in the emu thigh compared to the lower limb, more power is generated by the lower limb (Patak, 1988). Although it was expected the splayed-leg emus to have lower activities of metabolic enzymes and myoglobin concentration in their pelvic limb skeletal muscles, generally higher amounts were present.

Control limb

The GM is one of four bellies of the largest crus muscle in the emu pelvic limb (*M. gastrocnemius*) (Patak and Baldwin, 1998). The emu is the only known ratite to possess four bellies of the *M. gastrocnemius*, most have three. The GM is the large medial crus muscle that is responsible for ankle extension and knee flexion (Smith et al., 2006) (Fig. 3.2), and in many animals, this is a highly anaerobic muscle. There were no significant differences in LDH activity between the three muscles measured in the emu limb (Fig. 3.3). However, the GM had a lower myoglobin concentrations and lower activities of CS and HAD compared to the IFB and IFME (Fig. 3.3), suggesting the GM has less aerobic potential. Compared to the LDH activity in the hindlimb of the athletic dog (*Canis familiaris*), 396 ± 38 μmol min^{-1} g^{-1} wet tissue (Kanatous et al., 2002), the emu GM is over two-fold higher. The emu GM has similar aerobic capacity to that of the domesticated dog hindlimb muscle; the dog having a myoglobin concentration of 3.5 mg g^{-1} wet tissue, CS activity of 24.8 ± 3.3 μmol min^{-1} g^{-1} wet tissue, and HAD activity of 16.6 ± 2.8 μmol min^{-1} g^{-1} wet tissue (Kanatous et al., 2002).

The IFB is a muscle that is often analyzed in the hindlimb of birds (Marjoiniemi and Hohtola, 2000; McVean et al., 1987; Suman et al., 2010). In fact, the IFE was used in obtaining the amino acid sequence of myoglobin for emu skeletal muscle (Suman et al., 2010). This large

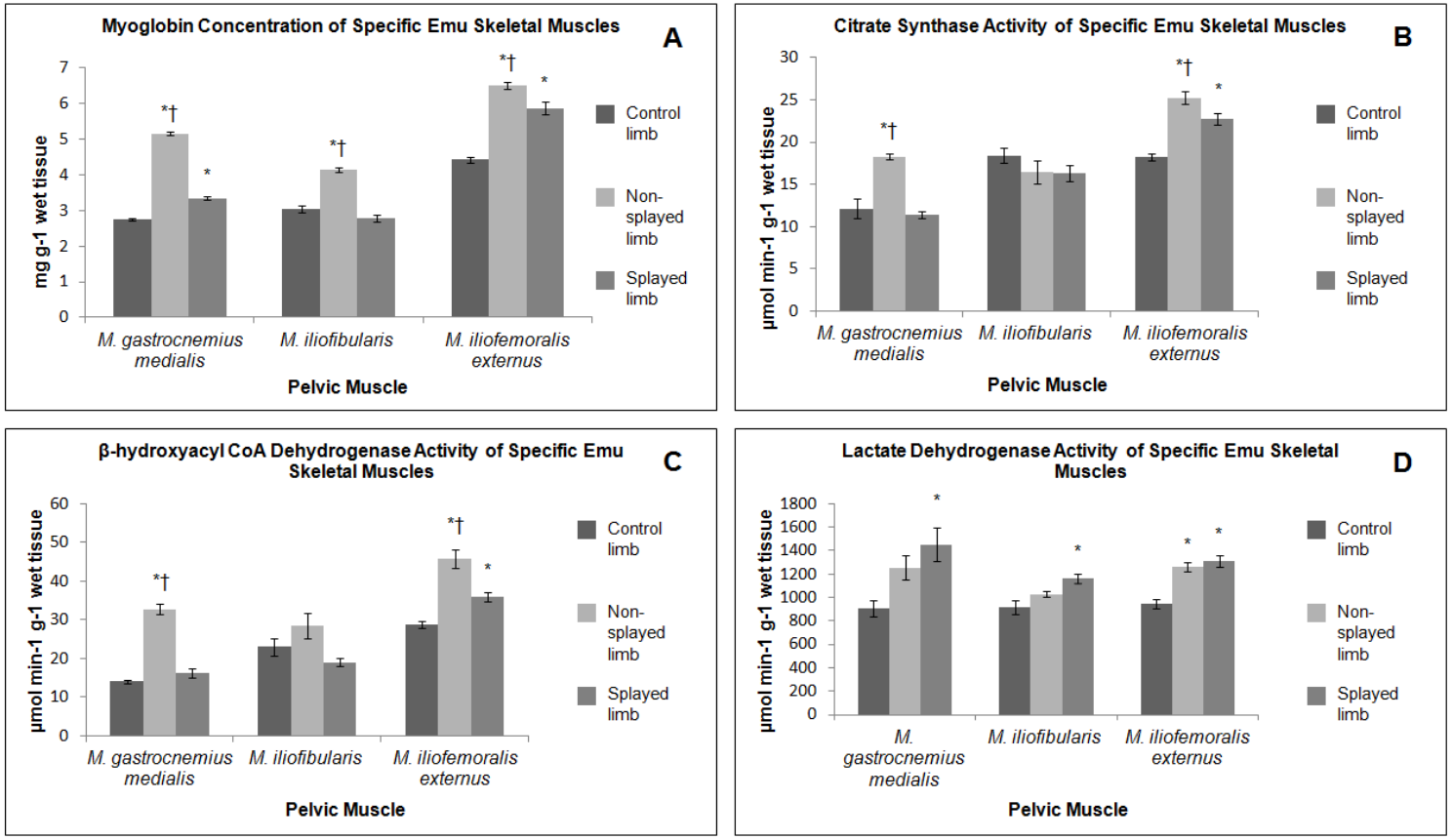

Fig. 3.3. Myoglobin (Mb) concentration (A), and enzymatic activities of citrate synthase (CS) (B), β-hydroxyacyl CoA dehydrogenase (HAD) (C), and lactate dehydrogenase (LDH) of specific pelvic limb skeletal muscles (*M. gastrocnemius medialis*, *M. iliofibularis*, and *M. iliofemoralis externus*). Four emus were included in this study. Three of these emus were healthy developing birds (control limb), and one emu suffered from a splayed-leg disorder. For the splayed limb individual, both the muscles of the non-splayed limb, and the splayed limb were analyzed. Myoglobin was measured in mg g^{-1} wet tissue and all enzymatic assays were measured in µmol min^{-1} g^{-1} wet tissue. All samples were run as triplicates, and each assay was repeated three times. ($P \leq 0.05$; means ± S.E.M).

***** Significantly higher than control limb
† Significantly higher than splayed limb

muscle is located on the lateral side of the thigh (Fig. 3.2). In ostriches this muscle is involved with knee flexion and hip extension (Smith et al., 2006). Because many similarities exist between ratite skeletal muscle anatomies (Patak and Baldwin, 1998), we assume the IFB has the same function in emus as it does in ostriches. The results suggest the IFB has a similar amount of aerobic and anaerobic capacity. The IFB has a myoglobin concentration more similar to the GM (a muscle with less aerobic potential), and a CS activity that resembles the IFME (a muscle with more aerobic potential) (Fig. 3.3). Also, the IFB has a HAD concentration that falls between that of the GM and IFME (Fig. 3.3).

The IFME is the pelvic limb muscle at the most proximal portion of the emu's thigh, and a tendon inserts this muscle to the lateral side of the femur (Patak and Baldwin, 1998) (Fig. 3.2). The IFME is responsible for hip adduction and flexion in the ostrich (Smith et al., 2006), and we assume the same function is performed by this muscle in emus. The IFME was the most aerobic of the three emu muscles analyzed, with the highest concentration of myoglobin and highest activity of HAD (Fig. 3.3). Once distal limb muscles like the GM tire, the proximal hindlimb muscles like the IFME become more important for sustained locomotion because of their enhanced aerobic characteristics.

Non-splayed and splayed limbs

Cooper et al. (2008) mentioned that pelvic limb muscles in domesticated ostriches (*Struthio camelus*) with bow leg syndrome (a similar orthopedic disorder to splayed-leg disorder) appeared to be emaciated and pale pink. It was assumed the emus would have weakened pelvic limb muscles that looked similar to these ostriches. Contrary to the prediction, no emaciation was noticed in splayed-leg emu pelvic muscles or a difference in color compared to healthy

birds. The skeletal muscles of the non-splayed and splayed limbs tended to be as high, or higher, in myoglobin content and metabolic enzymes activities compared to emu control limbs (Table 3.1). It is possible that bow leg syndrome acts differently upon ratite skeletal muscle than splayed-leg disorder. Also, the bow leg ostrich cases may have been more severe than the emu splayed-leg cases in this study. The splayed-leg emus do not spend much time sitting, unless it is a particularly severe case (T. L. Green, unpublished). This continued muscle stimulation may explain why the skeletal muscles did not decrease in myoglobin and metabolic proteins. Detraining occurs when an animal experiences a steep decline in exercise, and this can cause decreases in metabolic enzyme activity (Moore et al., 1987). In an attempt to keep up with other birds, splayed-leg emus will work their muscles harder, though the limping locomotion is less efficient. The non-splayed limb became much more aerobic due to this orthopedic stress (Fig. 3.3). This non-splayed limb is the limb that supports the majority of the emu's mass. Therefore, the muscles appear to work harder, thus upregulating aerobic proteins as a compensatory effect. Also, the CS:HAD ratio is lower in the non-splayed limb compared to the control limb, suggesting a higher reliance on β-oxidation to fuel aerobic metabolism (Table 3.1). It is likely the muscles in a normally-developing emu limb will have more time to relax. The splayed limb was, overall, more aerobically similar to that of a normal-developing emu limb (Fig. 3.3). The largest distinction between the control and splayed limb was in LDH activity. Because all three splayed limb muscles were significantly higher in LDH activity compared to the control limb, the splayed limb is significantly more anaerobic (Fig. 3.3). In a splayed-leg individual, this limb acts much differently than the non-splayed supporting limb. When a splayed-leg emu walks, the splayed limb spends little time on the ground. Similar to a human (*Homo* sapiens) with a limp, this limb moves forward quickly to prevent the animal from falling sideways. The more

repetitions a pathologic limb endures, the more anaerobic enzyme activity will be upregulated in the skeletal muscle.

The IFME muscle was selected for analysis because of its role in adduction of the hindlimb. It was assumed that this muscle in splayed-leg emus would look significantly different in metabolic profile compared to that of normal developing emus. IFME was the only muscle that was significantly higher in myoglobin concentration and all enzymatic enzyme activities in the splayed limb and non-splayed limb (Fig. 3.3). This illustrates that, of the three muscles analyzed, the IFME was affected by splayed-leg disorder the most, followed by the GM, and finally the IFB.

Domesticated and wild emus

A study conducted with Australian emu muscle found that wild emus have a slightly different metabolic profile than the domesticated emus from our study. For our study, the GM from healthy emus had an average myoglobin concentration of 2.74 ± 0.03 mg g^{-1} wet tissue, CS activity of 12.14 ± 1.21 µmol min^{-1} g^{-1} wet tissue, HAD activity of 14.05 ± 0.52 µmol min^{-1} g^{-1} wet tissue, and LDH activity of 905.44 ± 67.58 µmol min^{-1} g^{-1} wet tissue (Table 3.1). In the GM, wild emus had a myoglobin concentration of 4.7–13.3 mg g^{-1} wet tissue, CS activity of 50.7–62.8 µmol min^{-1} g^{-1} wet tissue, HAD activity of 3.4–6.8 µmol min^{-1} g^{-1} wet tissue, and LDH activity of 1471–2098 µmol min^{-1} g^{-1} wet tissue (Patak and Baldwin, 1993). Myoglobin, CS, and LDH were higher in wild emu GM tissue (Patak and Baldwin, 1993), which is expected, because naturally trained animals tend to have higher levels of myoglobin and metabolic enzyme activities (Butler and Turner, 1988). HAD activity was about 3-fold higher in the domesticated emus compared to the wild emus from Patak and Baldwin (1993). The domesticated emus

appear to metabolize lipids at a higher rate, though domesticated emus may eat a diet higher in fats than wild emus.

The general trend with these data illustrate that the splayed-leg emus have higher levels of myoglobin and metabolic enzymes in skeletal muscle tissue compared to control limbs (Fig. 3.3). Specifically, the non-splayed limb generally had higher activities of aerobic enzymes and levels myoglobin, and the splayed limb had relatively higher activities of anaerobic enzymes compared to the control limb (Fig. 3.3). Since these splayed-leg individuals have to work harder to keep the animal upright, the pelvic muscles actually look like those of a more athletic animal. Which element (bone, muscle, or tendon) of the limb that is defective cannot yet be determined, but the significant conclusion is that the pelvic limb muscles in the study were not initially weakened by the disorder.

Conclusions

This study was the first to classify metabolic profiles for domesticated emu skeletal muscles (control limb) (Table 3.1). It was found that the GM had less aerobic potential than the IFB and IFME, and the IFME had more aerobic potential than the GM and IFB (Fig. 3.3). Metabolic enzyme activities and myoglobin concentrations in the muscles of a disordered splayed-leg emu were also measured (non-splayed and splayed limb) (Table 3.1). The skeletal muscles tended to possess higher levels of myoglobin and metabolic enzyme activities (Fig. 3.3). Although the splayed-leg emus may walk less overall distance, the skeletal muscles work hard to keep the bird standing, and the grueling task causes those pelvic limb muscles to appear to belong to a more athletic, healthy animal. To compensate for the disorder, muscles in the non-splayed limb increased in aerobic potential, and muscles of the splayed limb increased glycolytic

potential. Because the non-splayed limb muscles have to contract more continuously, the muscles depend more upon oxidative metabolism. The splayed limb cannot support the full weight of the emu for much time, so it swings forward quickly during each stride, eventually causing the muscle tissue to become more glycolytically adapted. It can be concluded that emus in this study had no atrophy in the three muscles analyzed. If muscle atrophy occurs in splayed-leg emus, the condition has likely become severe enough that the animal cannot support its own body mass. To our knowledge, this is the first analysis of skeletal muscles from ratites with an orthopedic disease. Further inquiry as to why splayed-leg disorder develops will require more muscle and bone analysis, though the metabolic profiles from this study begin to shed light upon a common avian disorder.

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CHAPTER 4

Conclusions

Overview

Past studies have investigated emu physiology (Grubb et al., 1983; Patak, 1988; Patak and Baldwin, 1993; Maloney et al., 1994; Patak and Baldwin, 1998; Main and Biewener, 2007; Claessens, 2009; Suman et al., 2010), though most of this research has been conducted on wild emu muscle tissue (Patak, 1988, Patak and Baldwin, 1993; Patak and Baldwin, 1998; Main and Biewener, 2007). Because commercial farming of emus is so common in North America (Minnaar, 1998; American Emu Association, 2009), it is essential to understand how healthy emus develop, how they are metabolically fueled, and to investigate the pathologies and illnesses that exist. Emus are also an interesting model organism because they are impressive athletes, a large avian species, and flightless. Although the time period paleognathous birds lost the ability to fly is debated (Houde and Olson, 1981; Houde, 1986; Briggs, 2003; Harshman et al., 2008), it is agreed that ratites descended from a flighted relative (Briggs, 2003). Although flightless, flighted, and diving birds have different forms of locomotion, many physiological similarities still exist, including endurance capacity (Saunders and Fedde, 1994). By analyzing the physiological properties of emu muscle tissue, we aimed to set a physiologic baseline for farm-raised emu muscle tissue so future studies can determine if similarities exist between farm-raised, wild, and diseased emus.

This study was the first to classify the metabolic profile of ratite cardiac muscle. High concentrations of myoglobin and high activities of aerobic proteins suggest domesticated emus are predisposed to be elite avian athletes. It can be concluded that emu cardiac tissue is

inherently supported by these high myoglobin concentrations and high activities of metabolic enzymes during activity. Domesticated emu cardiac tissue is not metabolically similar to other farm-raised birds like chickens (Alp et al., 1976; Nishida, 1976; Butler and Turner, 1988). Instead, the metabolic characteristics of emu cardiac muscle are similar to active, flighted birds like pigeons and geese (Pages and Planas, 1983; Saunders and Fedde, 1991). Emus and aerobically active flighted birds share a number of similarities, including high levels of myoglobin and aerobic enzymes in cardiac tissue (Saunders and Fedde, 1994). Though emus are efficient sprinters and long-distance runners, they rely more directly upon aerobic metabolism to fuel activity.

This study was also the first to classify metabolic profiles for specific domesticated emu skeletal muscles. The *M. gastrocnemius medialis* (a large crus muscle) had less aerobic potential than the *M. iliofibularis* and *M. iliofemoralis externus* (both proximal thigh muscles), and of the three muscles, the *M. iliofemoralis externus* had the most aerobic potential. In measuring the metabolic enzyme activities and myoglobin concentrations in the muscles of an emu with splayed-leg disorder, it was found the skeletal muscles tended to be higher in myoglobin and metabolic enzyme activities in both limbs of the disordered animal. Although the splayed-leg emus may walk less overall distance compared to healthy animals, the skeletal muscles work hard as the bird staggers around and this grueling task causes those muscles to appear to belong to a more athletic, healthy animal. As a compensation for the disorder, muscles in the non-splayed leg increased in aerobic potential, and muscles of the splayed limb increased in anaerobic potential. This is consistent with the disordered emu's behavior, because the non-splayed limb muscles have to contract more continuously to support the animal's weight (dependence upon oxidative metabolism), and the splayed limb cannot support the full weight of

the emu for much time, so it swings quickly forward during each stride (dependence on glycolytic metabolism). It can be concluded that the emus in this study had no atrophy in the three muscles analyzed. If muscle atrophy occurs in splayed-leg emus, the orthopedic condition has likely become severe enough that the animal cannot support its own body mass at all. Further inquiry as to why splayed-leg disorder develops will require more muscle and bone analysis.

Future Directions

Emu ranching in the United States creates a unique opportunity for researchers to study large populations of exotic animals. Emu ranches can be found in almost every state in the United States (American Emu Association, 2009). By establishing the metabolic profile of farm-raised emu cardiac and skeletal muscle tissue, this study establishes a baseline for healthy emus, and allows for a number of potential projects to be conducted in the future. Investigation of the metabolic profile of skeletal muscle tissue of domesticated emus could be expanded to make comparisons between emus under different disease states, activity levels, diets, ages, sexes, and varying climate conditions.

Disease States

Splayed-leg is a common disorder in captive ratites (Gnad et al., 1996; Minnaar, 1998; Rothschild and Rühli, 2007; Cooper et al., 2008), though other orthopedic disorders are prevalent as well, including bowed legs, humped back, "knock knees", and twisted neck (Minnaar, 1998). It is undetermined if these different orthopedic disorders are caused by similar muscular or genetic defects. To better understand the anatomic or genetic causes of such conditions, as well

as prevention, the metabolic profile of every hindlimb muscle should be described from healthy and disordered emus. Also, emu bone and tissue histology samples should be analyzed in detail. The bone density should be compared between wild, captive, and disordered emus. This can illustrate if the animals are receiving the adequate proportions of minerals to maintain robust and strong bones.

Heart to Body Mass

Another measure that could give additional insight into overall cardiac health would be to measure the ratio between heart mass and body mass. The heart mass to body mass ratio has been recorded for numerous species throughout historic record and can be used as an assessment of health, and can also indicate myocardial disorders (Joseph, 1908). It would be interesting to see if emus with more lean muscle mass have a higher heart to body mass ratio than individuals with less lean muscle mass, or if splayed-leg emus have proportionally smaller hearts than emus without splayed-leg disorder. Because emu hearts are either used to supplement dog food or are thrown out at butchering establishments, whole hearts can be weighed and samples taken with ease.

Energetics

In this study a few of the characteristics that were not analyzed for potential differences were the metabolic characteristics between emu activity levels, diets, ages and sexes. To see whether particular pen sizes or the number of emus in a pen elicit metabolic changes, a full account of dimensional and behavioral data should be compiled from various ranches throughout the country. Most emus are fed a similar ratite chow, however variations occur between feed

companies, and this may cause differences in muscle tissue makeup. Most past studies have conducted experiments on emus between the ages of six to fifteen months of age. This is because most of the farm-raised emus are harvested right before, or right after they reach full size. This is the time samples can be collected easily. Farmers do not normally allow collection from young emus in fear of disrupting development, and do not want to sacrifice their older breeder birds. To better understand the full development of emus as they age, birds should be analyzed at a young age and after they reach sexual maturity, potentially by skeletal muscle biopsies. One unexplored area of emu (and other ratite) reproduction is what occurs physiologically to male emus during the breeding season. In the wild, and captivity, male emus incubate eggs almost continuously for 50 days (Minnaar, 1998). During this long incubation period the males rarely eat or drink, and it is unknown how their metabolism responds, prepares, or recovers from such a drastic decline in activity. It is possible that male emus decrease their metabolism accordingly and depend more on fat reserves during a particular season, like fasting flighted birds during long migrations (Blem, 1980; Dingle, 1996; Guglielmo, 2010). No current studies have investigated metabolic differences between emu sexes, especially in regard to these seasonal changes.

Ecology

By better understanding the metabolic demands of captive emus and other ratites, protocols can be established for studies with wild populations. Energetic studies are becoming more important for conservation biology projects, especially in predicting sustainable ranges and diets. More importantly, these metabolism studies are referred to when addressing political lawmakers about land management and natural resource uses. The ratite species that are of more

vulnerable conservation status, like the frugivorous cassowaries (*Casuarius sp.*) and the seclusive kiwis (*Apteryx sp.*) are particularly good candidates for extensive energetic studies. By denoting numerical approximations when discussing their behaviors, diets, and movements, biologists can give descriptive details regarding population health to colleagues, politicians, and the general public.

Breeding

No major differences were observed in metabolic protein activity between the two ranches, despite slight elevation differences. However, breeding success can be impacted by moving emus from ranch to ranch (T. L. Green, unpublished results), though it is unclear whether elevation variations are the detrimental factor. For emu hatchlings, hypoxia (low-oxygen environment) has been shown to have an effect on cardiac processes (Shah et al., 2010). Though heart rate was unchanged, significant differences in β-adrenergic tone existed between hatchling emus under normoxic and hypoxic environments (Shah et al., 2010). Elevation differences have also been shown to have an effect on other vertebrate groups (Esteva et al., 2009). In previous studies with rat myocardium, it was determined that there were significant changes in myoglobin concentration, CS activity, and LDH activity due to simulated elevation differences (Esteva et al., 2009). To better understand the effects of elevation on farm-raised emus, future studies should focus upon birds raised at sea level to see if physiological differences exist between them (a baseline population) and high elevation populations.

Ontogeny

Additionally, the evolutionary history and anatomy of emus make them excellent models for branching into comparative studies with early birds and theropod dinosaurs (Padian and Olsen, 1989; Castanet et al., 2000; Schweitzer et al., 2005; Milàn, 2006; Breithaupt et al., 2007). Emus exhibit a fast period of growth after hatching from eggs (Minnaar, 1998; Breithaupt et al., 2007). The combination between this rapid development, skeletal similarities, and the parental care exhibited by adult male emus (Minnaar, 1998), make them excellent modern analogs for paleontologists attempting to reconstruct theropod dinosaur development, growth, and behavior. Full osteological growth records and dimensional data can be taken from emus and compared to data sets complied from theropod dinosaurs. Many theropod dinosaurs appear to have grown slower than ratites (Erickson et al., 2007), though this can be compensated for in growth estimations.

Conclusions

Avian physiology is fascinating to researchers because birds are metabolically equipped to undergo long flights, dives, and runs. However, limited skeletal muscle physiology has been conducted on the flightless ratites (Grubb et al., 1983; Patak, 1988; Patak and Baldwin, 1993; Maloney et al., 1994; Patak and Baldwin, 1998; Main and Biewener, 2007; Claessens, 2009; Suman et al., 2010), and even fewer studies have focused on ratite cardiology (Grubb et al., 1983; Kato et al., 2002; Moriya et al., 2002; Shah et al., 2010). These studies classify the metabolic profiles of farm-raised emu cardiac and skeletal muscle tissue for the first time. The collected data illustrate the similarities in cardiac metabolism between ratites and active, flighted birds. The pelvic limb muscle data established a baseline metabolic profile for specific farm-raised emu skeletal muscles, and also illustrate differences between healthy emus and those

affected by splayed-leg disorder. Building a physiological picture for farm-raised ratites is essential to the understanding of this unique model species.

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