PLASMA ß-HYDROXYBUTYRATE AND FLIGHT IN *EPTESICUS FUSCUS*

THE EFFECT OF FLIGHT DURATION ON ß-HYDROXYBUTYRATE CONCENTRATION IN BLOOD PLASMA OF *EPTESICUS FUSCUS*

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A Thesis Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements for the Degree Master of Science

McMaster University MASTER OF SCIENCES (2020) Hamilton, Ontario (Psychology)

TITLE: Effect of Flight Duration on ß-hydroxybutyrate Concentration in Blood Plasma of *Eptesicus fuscus*

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NUMBER OF PAGES: ix, 34

Lay Abstract

The “fasting while foraging” hypothesis states that the metabolic demands of flight can exceed energy intake from recently consumed prey items, so insectivorous bats may metabolize fat stores (especially early in the night) to power flight with ketones, a byproduct of the normal oxidation of fatty acids. Previous studies in bats have found increases in the plasma ketone ß-hydroxybutyrate following food consumption, but no study has explored whether increases in plasma ß-hydroxybutyrate occur following flight without food consumption. We collected and analyzed blood to examine changes in plasma ß-hydroxybutyrate following different flight durations in big brown bats. We explored both seasonal and captivity effects. To explore seasonal effects, we sampled blood from bats in the fall and the spring, times that are biologically significant to big brown bats. The spring is when bats move out of torpor, a form of hibernation, into an active state and the fall is when bats are preparing for entering into torpor. To explore captivity effects, we sampled blood from bats recently introduced to or established to captivity. Bats were fasted for 12 hours prior to flight (exercise treatment) or rest (control), and then blood was collected. We characterized exercise using flight time. We found that plasma ß-hydroxybutyrate increased after longer flight durations, which supports the fasting while foraging hypothesis.

Abstract

Insectivorous bats alter relative use of metabolic substrates to match requirements of their activities, including energetically expensive flight. The "fasting while foraging" hypothesis states that the metabolic demands of flight often exceed energy intake while foraging, hence bats may metabolize fat stores (especially early in the night) to power flight with ketones, a byproduct of the normal oxidation of fatty acids. Previous studies in bats have found increases in the plasma ketone ß-hydroxybutyrate following food consumption paired with or without flight. However, no study has explored whether increases in plasma ß-hydroxybutyrate occur following flight without food consumption. We used metabolite analysis to examine changes in plasma ß-hydroxybutyrate as a function of flight duration in 2 groups (fall and spring) of captive big brown bats (*Eptesicus fuscus*). We fasted bats for 12 hours prior to flight (exercise treatment) or rest (control), and then collected interfemoral vein blood. Exercise activity was quantified as flight time. For the Fall group, we collected three rest samples and one flight sample. Results for the Fall group were variable; interpretation of data patterns for this group may be complicated by changes in metabolism that occur in the fall when bats physiologically prepare for hibernation. To control for seasonal effects, we tested a second group of bats in the spring, collecting two rest and three flight samples. We found a positive correlation between flight duration and levels of plasma ß-hydroxybutyrate in the Spring group, which supports the fasting while foraging hypothesis.

Acknowledgements

I would like to thank my supervisor, Dr. Paul A. Faure, for supporting me through the past two years and assisting in helping create meaningful research collaborations, and my co-supervisor, Dr. Deda C. Gillespie, for always encouraging increased scientific rigor. I also thank Dr. Nicholas Bock and Dr. Ian Bruce (Department of Electrical and Computing Engineering) for serving on my Supervisory Committee. I would like to thank my collaborators, Dr. Liam P. McGuire and Dr. Justin G. Boyles, for allowing me the opportunity to work on this experiment and use it as my M.Sc. thesis.

I also wish to thank all the members of the McMaster University Bat Lab who have helped me during the past two years, and Dr. Kathleen Delaney, Ms. Dawn Graham and the staff of the Central Animal Facility staff for their continuous care of our bats.

I would like to extend a special thank you to the undergraduate students who assisted me with flying bats: Emily Wuerch, Grace Huang, and Shane Seheult.

I am grateful for having had the opportunity to learn a variety of new techniques and to explore new research topics in the McMaster Bat lab.

Finally, I would like to thank my family for always supporting me in my ambitions and endeavors.

Semper ad meliora

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β-hydroxybutyrate	Beta-Hydroxybutyrate
Acetyl-CoA	Acetyl-Coenzyme A carboxylase

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Introduction

Mammalian species utilize different types of metabolism depending on a variety of factors, which can be summarized as energetic input versus energetic demand. Two key types of metabolism are carbohydrate catabolism and lipid catabolism (Secor, S. M., & Carey, H. V., 2011; Novello, F., Gumaa, J. A., & McLean, P., 1969; Gumaa, K. A., Novello, F., & McLean, P., 1969). Catabolism refers to the process of breaking down molecules into various byproducts that can be used in oxidation or anabolic reactions. Anabolic reactions involve the synthesis of new molecules, which requires energy. Animals typically follow a pattern of food consumption based on species, but general metabolic processes exist (Secor & Carey, 2011). Animals consume food, which is then digested through the use of enzymes to break the food down into fundamental components such as amino acids, monoglycerides, and fatty-acids. These fundamental components are dispersed throughout the body and used in a series of metabolic reactions to fuel various activities. The types of metabolic reactions that occur are a result of which fundamental components, such as monoglycerides or fatty-acids, are present to commence metabolic reactions. The availability of each product also limits subsequent metabolic reactions.

When a mammal consumes carbohydrate-heavy food, it catabolizes carbohydrates to produce fuel for energetic demands (Novello et al., 1969; Gumaa et al., 1969). Alternatively, when a mammal consumes a low amount of carbohydrate-based food or no food, its body supplies energetic demands through lipid catabolism (Rakvaag, E., Lund,

M. D., Wiking, L., Hermansen, K., & Gregersen, S., 2019; Secor & Carey, 2011; Poesen, R., Mutsaers, H. A., Windey, K., van den Broek, P. H., Verweij, V., Augustijns, P., ... & Meijers, B., 2015; McGarry, J. D., Meier, J. M., & Foster, D. W.,1973). Expected use of these two metabolic paths differs depending on diet, natural fasting bouts, and physical activity level (Secor, & Carey, 2011; McGarry et al.,1973). Some mammalian species do not consume enough carbohydrates to supply their energetic demands through carbohydrate catabolism, and thus rely on lipid catabolism. One example of such a mammal that eats next to no carbohydrates is the common vampire bat (*Desmodus rotundus*). Depending on the ratio of energetic input to demands at a given time, some mammals may alternate between metabolic approaches. An example of this occurs in sleeping humans. During sleep, when they are not consuming new carbohydrates, humans generally rely on lipid catabolism rather than carbohydrate metabolism (Robinson, A. M., & Williamson, D. H., 1980). Although, these two metabolic approaches follow different biochemical pathways both ultimately result in the production of ATP, which is then used as a source of energy (Robinson & Williamson, 1980).

During both metabolic pathways, different reactions occur, producing various by-products used in subsequent reactions. As by-products of metabolic pathway reactions, metabolites can be used to measure energetics (Rakvaag et al., 2019; McGarry et al.,1973). For example, because carbohydrate and lipid catabolism produce different metabolites, researchers can analyze the levels of certain metabolites, to determine which form of catabolism is being used and thus to infer the animal's recent energetic demands (Rakvaag et al., 2019; Franklin, M. P., Sathyanarayan, A., & Mashek, D. G., 2017; McGarry et al.,1973). Standard metabolites used for such analysis of metabolism are:

triglycerides, beta-hydroxybutyrate, cholesterol, acetoacetate, and lactic acid (Rakvaag et al., 2019; Jenni-Eiermann, S., & Jenni, L., 1994; McGarry et al.,1973). Different metabolite analysis should be conducted for different sample types, as metabolites are produced and transported in different ways. Beta-hydroxybutyrate and acetoacetate are an example of two metabolites that indicate fatty-acid catabolism but would be present in different concentrations in blood and urine (Galvin, R. D., Harris, J. A., & Johnson, R. E., 1968; Yu et al., 2011). In the case of β-hydroxybutyrate, nonesterified fatty acids in the liver are converted into Acetyl-Coenzyme A carboxylase (Acetyl-CoA) when exposed to beta oxidation. The Acetyl-CoA is then used in the Krebs cycle to produce energy in the form of ATP, which can be used for various cell functions. Acetyl-CoA is also a precursor for ketone body production (Laffel, L., 1999; Robinson & Williamson, 1980). Ketone bodies are a family of molecules that include acetoacetate and β-hydroxybutyrate. Ketone bodies are released from the liver into the blood stream, where they are then transported to cells in the body. Ketone bodies are a precursor for Acetyl-CoA synthesis (Laffel, L., 1999).

Energetic demands result from the combined basal metabolic requirements and physical activity of an individual animal. Basal metabolism encompasses all the energetic needs of the body to function without added activity and largely includes the metabolic needs of organs to operate to maintain life (White, C. R., & Seymour, R. S., 2005; Weibel, E. R., & Hoppeler, H., 2005; McNab, B. K., 1997). Physical activity is an additional energetic demand that is required of mammals to procure food and survive (Weibel & Hoppeler, 2005). Changes in metabolic needs occur throughout the day as activity levels increase or decrease. In the case of bats, active flight requires more energy

than roosting, so bats adjust their metabolism to meet the energetic demands of their current activity.

Insectivorous bats require changes in metabolic activity to compensate for high-energy activities such as flight (McGuire, L. P., Fenton, M. B., & Guglielmo, C. G, 2009). Metabolite analysis in wild animal populations is commonly used to assess energetics and activity (Williams, T. D., Guglielmo, C. G., Egeler, O., & Martyniuk, C. J.,1999; Whittier, J. M. & Mason, R. T.,1996). Multiple factors contribute to concentrations of plasma metabolites in blood, making it is difficult to assess which factors have the most influence on plasma metabolite concentrations (McGuire et al., 2009a; McGuire, L. P., Fenton, M. B., Faure, P. A., & Guglielmo, C. G., 2009; Rojas-Morales, P., Tapia, E., & Pedraza-Chaverri, J., 2016). For example, blood plasma concentrations of the ketone beta-hydroxybutyrate (β-hydroxybutyrate) can provide information regarding metabolic refueling intensity and reflect changes in body mass, food consumption, and/or recent activity (McGuire et al., 2009a; McGuire et al., 2009b; Rojas-Morales et al., 2016; McGarry et al.,1973). Mammals appear to have a species-specific baseline range of β-hydroxybutyrate levels. This baseline range exists for a number of reasons. For example, in humans most of our baseline β-hydroxybutyrate plasma concentration is produced while we sleep to continue regular body functions (Robinson & Williamson, 1980). Deviation from the species-specific normal range, can be lethal. In humans, deviations in normal ketone ratios can result in type 2 diabetes and/or ketoacidosis (Laffel, L., 1999). Other mammalian species can also enter ketoacidosis through deviations in ketone ratios as a result of consuming too little carbohydrates and/or excessive physical activity (Rakvaag et al., 2019; McGarry et

al.,1973). The assessment of changes in plasma ß-hydroxybutyrate may also provide new insights for assessing the activity levels of individual animals, thus furthering our understanding of bat metabolism and mammalian physiology.

Insectivorous bats employ a variety of flight strategies to capture prey while foraging, which could help to optimize the use of metabolic fuel and meet the demands of energetically expensive flight. Examples of two flight foraging strategies are: (1) sit-and-wait foraging, where the bat waits at a perch for prey items to pass by before employing the technique of sallying out from its perch with a short-burst of flight to catch the insect and then returning to the perch (same or different) to consume the prey, and (2) aerial-hawking foraging, where a bat searches for and captures insect prey in continuous flight.

Big brown bats (*Eptesicus fuscus*) in North America mostly employ an aerial-hawking foraging strategy (Arbuthnott, D., & Brigham, R. M., 2007; Kurta & Baker 1980; Surlykke and Moss 2000). Aerial-hawking bats have been reported to probabilistically hunt multiple prey items at the same time (Fujioka, E., Aihara, I., Sumiya, M., Aihara, K., & Hiryu, S., 2016). By tracking multiple prey with echolocation, bats may be able to more efficiently capture prey and maximize food consumption while minimizing physical exertion and reducing flight time. While the exact daily energetic needs of *E. fuscus* have not been reported, the estimated energetic requirement to maintain current mass at 20°C is 0.22Kcal/g of animal mass per day, which is a lower calorie-to-mass ratio than other insectivorous bats. For example, a 15g *E. fuscus* would require 3.3 Kcal to maintain its current 15g mass. The little brown bat (*Myotis lucifugus*) is another aerial-hawking species that requires an average of 26.4 kJ per day for an average mass of 7.5g (Boyles et al., 2016), which is 3.52 kJ/g per day. These estimated

energetic requirements can be converted to an equivalent unit using a conversion rate of 1 kilocalorie being energetically equal to 4184 Joules. This results in the little brown bat requiring approximately 0.84Kcal/g per day. The big brown bat has a much smaller caloric requirement per gram than the little brown.

There can be a trade-off between storing excess lipids that can be metabolized later *versus* the additional energy that must be expended to accommodate flying with a higher body mass due to stored fat. Active flying is energetically demanding and puts considerable strain on a bat. Previous researchers have calculated that a 7.5 g *M. lucifugus* would use 4.14 kJ/h while flying and would forage for approximately 2 hours a night (Boyles et al., 2016). Flying at higher altitudes and/or over longer distances can make flight more energetically efficient than other types of locomotion. However, at short distances flight is an energetically inefficient mode of locomotion. The start or initiation of active flying can be energetically demanding, especially before any prey has been captured.

Metabolite analysis is routinely used to assess recent activities of wild animals, including food consumption and fat catabolism. Some metabolites that are often used to assess energetics are β-hydroxybutyrate and triglycerides (McGuire et al., 2009; Williams, T. D., Guglielmo, C. G., Egeler, O., & Martyniuk, C. J., 1999; Whittier, J. M. & Mason, R. T., 1996). Blood plasma increases or decreases in the ketone β-hydroxybutyrate can be used as a proxy measure for fat catabolism and determining if an animal is mobilizing fat stores to supply metabolic energy (Rakvaag et al., 2019; Puchalska, P., & Crawford, P. A., 2017; Price, E. R., Jones, T. T., Wallace, B. P., & Guglielmo, C. G.,2013; Andrews, M. T., Russeth, K. P., Drewes, L. R., & Henry, P. G.,

2009). In mammals, plasma triglycerides increase following food consumption, hence they can be used as a proxy measure of recent feeding activity (Franklin et al., 2017). β-hydroxybutyrate and triglycerides have a negative correlation to each other and provide a snapshot of either recent feeding or use of fat reserves in wild populations. The negative correlation between β-hydroxybutyrate and triglycerides is not limited to mammals as it also occurs in avian (Jenni-Eiermann & Jenni, 1994; M. Zajac, R., J. Cerasale, D., & G. Guglielmo, C., 2006; Jenni-Eiermann, S., Jenni, L., Kvist, A., Lindström, Å., Piers., & Visser, G. H., 2002) and reptilian species (Price et al., 2013; Whittier, J. M., & Mason, R. T., 1996). Recent studies in insectivorous bats have reported a positive correlation between the plasma concentration of β-hydroxybutyrate and triglycerides (McGuire et al., 2009a; Boyles et al., 2016; Baloun, D. E., Webber, Q. M., McGuire, L. P., Boyles, J. G., Shrivastav, A., & Willis, C. K., 2019).

The "fasting while foraging" hypothesis was recently proposed by Baloun *et al.* (2019) to explain some of the variance that has been observed in previous studies on insectivorous bats in plasma metabolite concentrations between individuals and across different experiments. This hypothesis proposes that the metabolic energy demand for flight is higher than what bats can consume in insect prey initially after leaving the roost. The high energetic demand of flight results in a similar physiological response as fasting. A previous study on the lesser bulldog bat (*Noctilio albiventris*) attempted to separate what component of flight was fueled by the energy of recently consumed insect prey *versus* from catabolized lipid stores, and found that it takes approximately 60 minutes for energy from a consumed insect to be available as fuel for flight (Voigt et al., 2010). A delay of 60 minutes for consumed food to be digested and converted into cellular energy

implies that insectivorous bats need to have at least 60 minutes of flight fuel available from lipid stores prior to the commencing of foraging.

When an animal catabolizes fat stores into energy, ketone production increases and ketones enter the bloodstream (McGarry et al.,1973). These newly made ketones are transformed through multiple biological pathways into ATP, which supports the function and energetic demands of tissues (Andrews et al., 2009). Interestingly, in big brown bats, food consumption can also result in higher blood concentrations of both ketones and triglycerides (McGuire et al., 2009b). Most mammalian species have a negative correlation between ß-hydroxybutyrate and triglyceride blood concentrations (Baloun et al., 2019). A further study found that both ß-hydroxybutyrate and triglyceride plasma concentrations have a negative correlation for increased food rate consumption, quantified as the time required for the bat to consume a set insect mass (Baloun et al., 2019). The trend suggests that other molecular pathways may be involved and driving the positive correlation that was observed between these two plasma metabolites in big brown bats (McGuire et al., 2009b).

In this study, we measured plasma β-hydroxybutyrate in big brown bats from a captive research colony so we could re-sample the same individuals and examine changes in plasma β-hydroxybutyrate pre- and post-physical activity. We did this by collecting blood samples from rested and exercised bats that were fasted. We quantified exercise activity as flight duration. Our data indicate there was a positive correlation between flight duration and plasma ß-hydroxybutyrate.

Methods/ Materials

Study Animals

The study consisted of three groups of big brown bats (*Eptesicus fuscus*). Two of the groups were recently captured animals that were new to captivity and the third group consisted of bats that had been established for 1-3 years in captivity (Table 1). The three experimental groups were sampled during different seasons. The first group was captured in August 2018 and their blood sampled during late October to early December 2018 (Fall group). The second group was captured in May 2019 and their blood was sampled from June to July 2019 (Spring group). The third group consisted of animals who had resided in captivity for at least one calendar year before blood sampling. This group was sampled from July to September 2019 (Established group).

At McMaster bats were housed in an indoor, free-flight facility (2.5 x 1.5 x 2.3 m; l × w × h) where the colony temperature and lighting varied with ambient conditions (Skrinyer et al., 2017). Except during testing, bats were given *ad libitum* access to food (mealworms, *Tenebrio molitor*) and water. Individuals were identified by numbered plastic or metal forearm bands and a subcutaneous passive integrated transponder (PIT) tag (Trovan ID100A, EIDAP Inc.). When not tested, bats were housed together in stainless steel (1/4" mesh) holding cages (28 × 22 × 18 cm) in a temperature and humidity-controlled holding room in the Psychology Animal Facility. All experimental procedures conformed to the guidelines for the care and use of wild mammals in research published by the American Society of Mammalogists (Sikes et al. 2011), and care and use of experimental animals published by the Canadian Council on Animal Care, and were approved by the Animal Research Ethics Board of McMaster University.

Experimental Design

Over the course of a year, fourteen newly captured adult bats were selected and randomly assigned to a cage in the McMaster Bat Lab Colony. These fourteen bats were used in the Fall (n=9) and Spring groups (n=5). Additionally, five adult bats established to captivity in the McMaster Bat Lab Colony were selected and randomly assigned to a cage for the Established group.

Animal Housing

Animals were housed in groups of 3-5 bats for the duration of the experiment and fasted for 12 hours prior to each blood collection (Figure 1.a). The first group of bats were housed in groups of 3. The second group of bats were housed in either individually or in a cage of 3. The third group of bats, established to captivity, were moved from the colony into a cage in the behavioural room of the McMaster Bat Lab during July 2019 (Table 1). All animals had *ad libitum* access to water throughout the experiment and all animals had *ad libitum* access to food except when fasting prior to a blood sample collection.

Flying Bats

Bats were encouraged to fly in a room with anechoic foam lined walls located in the basement of the Psychology Animal Facility. The bats were chased by 2-4 colleagues throughout the room for the duration of the pre-determined flight time to ensure they were actively flying. Ladders were used to reach bats that had landed on the walls. Once collected, bats were forced to fly again. Flight durations are approximate and every effort was made to ensure that the bats flew for the majority of the reported flight durations.

Bats who landed frequently were forced to continue flying until they approximately reached the reported time.

Blood samples were collected in the same order for all groups; the first rest sample being first, followed by samples from flight durations in descending order, and the second rest sample being conducted last (Figure 1.b). One animal from the Spring 2019 group did not conduct all flight durations in this order; as it refused to fly the evening of the 5 minute flight collection, we collected a second rest blood sample instead. This animal died prior to collecting the 5 minute flight blood sample.

Blood Collection

Bats were restrained using a custom restrainer (Ceballos-Vasquez, A., Caldwell, J. R., & Faure, P. A., 2014) and then bled from the interfemoral vein with a sterile 27-gauge needle (Becton, Dickinson and Company, Franklin Lakes, NJ) (Figure 1.c). Blood was collected using 100 µL micro-hematocrit tubes (Fisher Scientific, Pittsburgh, PA) and then transferred into a clean PCR tube (Fisher Scientific, Pittsburgh, PA) that was centrifuged at 2000Gs for 10 minutes (Figure 1.d-e). The volume of blood collected ranged from approximately 60 to approximately 200 µL. To minimize differences in collection time and animal stress from handling, all blood samples were collected and stored individually prior to beginning the centrifuging process. Afterwards, 10 µL of plasma from the centrifuged tube was pipetted onto a commercially available and experimentally validated (Sommers, A. S.; Boyle, W. A.; & McGuire, L. P., 2017) test strip (STAT-Site M b-HB Photometer, part number 400400; Stanbio Laboratory, Boerne, TX) and analyzed for plasma ß-hydroxybutyrate concentration (Figure 1.e-f). Bat mass

was recorded prior to returning the animal to its designated cage. All blood draws occurred between 8 pm and 9 pm.

Fasting

To ensure all animals were in a fasted state, bats were fasted for 12 hours the day of the sample collection. Animals were allowed *ad libitum* access to water throughout the experiment. To fast the bats, food dishes were removed from the cages 12 hours prior to starting the experiment.

Experiment 1 – Fall 2018 Group (Between- Subjects Design)

Animals were housed in groups of 3 for the duration of the experiment and allowed *ad libitum* access to food between experimental trials. Fasted and rested bats were either flown for a set duration of time or were immediately restrained and bled. The durations of flight were 5, 8, or 10 minutes. Every cage had a bat randomly assigned to each of the flight duration categories, for a total of 1-3 animals in each flight duration category. Each animal completed one flight time and 3 rest trials (Table 1). All samples were collected one week apart to allow the animals time to recover from being bled.

Experiment 2 – Spring 2019 Groups (Within- Subjects Design)

Animals were housed in groups of 1 or 4, separated by capture site, for the duration of the experiment and allowed *ad libitum* access to food between experimental trials. Bats were either flown for a set duration of time or were immediately restrained and bled. The durations of flight were 5, 10, and 15 minutes. All animals completed the three durations of flight and two rest trials (Table 1). All samples were collected one week apart to allow the animals time to recover from being bled.

Experiment 3 – Established to Captivity Group (Within- Subjects Design)

Animals were housed together for the duration of the experiment and allowed *ad libitum* access to food between experimental trials. Bats were either flown for a set duration of time or were immediately restrained and bled. The durations of flight were 5, 10, and 15 minutes. All animals completed the three durations of flight and two rest trials (Table 1). All samples were collected one week apart to allow the animals time to recover from being bled.

Data Analysis

Unless stated otherwise, all data are reported as the mean ± standard deviation (SD). A repeated measures ANOVA was used to analyze the concentration of ß-hydroxybutyrate after different flight durations in the Spring and Established groups. This analysis was conducted using SPPS Statistics, Version 25.0 (IBM, Armonk, New York). Pearson's correlation data analyses were used analyze the concentration of ß-hydroxybutyrate after different flight durations in the Fall group. Pearson's correlation analyses and box and whisker plots were completed using R Version 3.6.1 (RStudio, Inc., Boston, MA). In the Box and whisker plots the bold line in the box represents the mean. The bottom and top of the box represent the 25th and 75th percentiles. The interquartile range (IQR) is the 75th percentile minus the 25th percentile. The bottom and top whiskers represent the minimum (first quartile minus 1.5 times IQR) and the maximum (third quartile plus 1.5 times IQR), respectively; the open circles are data values that fall below and/or above these values.

The Spring and Established groups where analyzed using a repeated measures ANOVA and Pearson's correlation, while the Fall group was analyzed using Pearson's correlation.

Samples that did not produce 10 µL of plasma were recorded but not included in the final data analysis. Some bats were re-sampled for time points when not enough whole blood was collected to produce 10 µL of plasma. One animal from the Spring 2019 group was not included in the repeated measures ANOVA for not having samples for all flight time durations. We were not able to collect all flight durations from that animal as it died prior to completion of the experiment.

Results

Study Animals

I obtained plasma ß-hydroxybutyrate concentrations from 19 individual big brown bats. The average bleed time was 3.016 ± 0.059 min (range: 0.666–8.066 min).

Experiment 1 – Fall 2018 Group (Between-Subjects Design)

Each bat in the Fall 2018 group had only one flight duration, so no conclusions or observations can be made regarding changes in flight or landing behaviour in individuals as a function of flight duration. Most noticeably, the Fall 2018 bats rarely landed on the walls of the behavioural testing room when they were flown. Bats in the 10 minute group landed a few times; however, the single 15 minute flight bat did not try to land until after 14 minutes of continuous flight.

Plasma ß-hydroxybutyrate concentrations measured from bats in the Fall 2018 group were significantly correlated to flight time, $r(25)=0.617$, $p=0.0006$ (Figure 2 and Figure 4). During the sampling period for the Fall 2018 group, plasma ß-hydroxybutyrate

concentrations in the rested conditions first increased but then became more variable with each subsequent blood sampling collection.

Experiment 2 – Spring 2019 Groups (Within-Subjects Design)

Observationally, the bats' flight behaviour changed throughout the duration of the experiment. Most noticeably, the Spring 2019 bats rarely landed on the walls for the first few flight durations; however, by around sample collection 3, they started to frequently land on the walls. The time to catch the landed bats also increased because the animals quickly learned how to crawl behind the anechoic foam lining the walls of the room and this made it harder for the human observers to collect them. Observationally, the bats displayed more aggressive biting and general evasion behaviour towards human handlers involved in the experiment as sample collection progressed. These aggressive behaviours were not observed with other handlers or when the bats were allowed *ad libitum* access to food. This may suggest that they learned when blood samples would be collected based on the availability of food.

Plasma ß-hydroxybutyrate concentrations measured in the Spring 2019 group were not significantly correlated with flight duration, $r=0.3105$, $p=0.149$.

Experiment 3 – Established to Captivity Group (Within-Subjects Design)

The bats that were established to captivity did not show any obvious changes in the frequency of wall landing over the course of their sample collections. During the flight durations, these bats landed on a wall every couple of minutes. As with the bats in the Spring 2019 group, the time to catch bats that had landed on the wall increased as the bats learned how to crawl behind the anechoic foam lining the walls of the room which made it harder for the human observers to find and collect them. Bats tested in this group

were familiar with the behavioural flight room prior to conducting the experiment and consequently may have developed preferences for particular landing and hiding spots in the room prior to the experiment.

Plasma ß-hydroxybutyrate concentrations in the Established group were significantly correlated to flight time, r=0.685, p=0.00015.

Between-Group Comparisons

Plasma ß-hydroxybutyrate concentrations in the Spring 2019 and the Established groups were significantly correlated to flight time, $r(47)$=0.408, p=0.002, and group $r(47)$=0.358, p=0.006 (Figure 5 and Figure 6). Additionally, a within-subject one-way repeated measures ANOVA showed that the plasma ß-hydroxybutyrate concentrations differed significantly between flight duration samples for the pooled Spring 2019 and Established group data (F(2.687, 21.496) =3.298, p= 0.044). A Shapiro-Wilk normality test was conducted to test for normality of the pooled Spring 2019 and Established group dataset (W=0.983, p=0.688). The result of the Shapiro-Wilk normality test indicate that the data are normally distributed. Mauchly's test for sphericity indicated that the pooled data from the Spring 2019 and Established groups had no significant difference in variance between flight durations (Mauchly's W = 0.235, p= 0.427). However, a small sample size can alter the results of a sphericity test, so the Greenhouse-Geisser estimate (ϵ=0.672) was used to correct the degrees of freedom in the one-way repeated measures ANOVA. Plasma ß-hydroxybutyrate concentrations in the Fall 2018 group were also significantly correlated to flight time, $r(25)$=0.617, p=0.0006 (Figure 2 and Figure 3). Finally, plasma ß-hydroxybutyrate concentrations in all three groups were significantly correlated $r(74)$= 0.382, p=0.00065.

The results indicate a pattern of increased plasma ß-hydroxybutyrate concentrations for longer flight durations $r(74)= 0.382$, $p=0.00065$. Bats in the Established group that had spent at least one year in captivity showed a stronger correlation between plasma ß-hydroxybutyrate levels and flight time (r=0.685) than the recently captured bats from the Fall 2018 (r=0.617) and Spring 2019 (r=0.3105) groups. Animals did have individual variation in plasma ß-hydroxybutyrate concentrations. Some animals had increased concentrations after longer flights while some had decreased concentrations, and this appeared to be especially true for the Spring group (Figure 6, Figure 7).

Discussion

Concentrations of plasma ß-hydroxybutyrate increased with flight duration across all groups. The Fall group had a significant positive correlation between plasma ß-hydroxybutyrate concentrations and flight duration (r=0.617). Additionally, some animals in the Fall group had a pattern of increasing rested condition plasma ß-hydroxybutyrate concentrations with each rest sample. The rest blood samples for the Fall group also had more variability in plasma ß-hydroxybutyrate concentrations than the other groups (Figure 3). The Spring group had a positive correlation between plasma ß-hydroxybutyrate concentrations and flight duration (r=0.3105). The Spring group had the lowest correlation between plasma ß-hydroxybutyrate concentrations and flight duration. The Established group had a significant positive correlation between plasma ß-hydroxybutyrate concentrations and flight duration (r=0.685). The Established group had the highest correlation between plasma ß-hydroxybutyrate concentrations and flight duration.

The non-random animal selection paired with small animal numbers could explain the variation in correlation between the Established bats and the Fall and Spring groups. Animals were pre-selected for the study based on sexual status (no pregnant or lactating females were included), and flying ability. The criteria, in particular flying ability, for participation in the experiment could result in inherent biases and variation. Bats that were able to continuously fly for upwards of fifteen minutes after periods of captivity may have higher levels of activity when housed in small cages than bats who are unable to fly for upwards of fifteen minutes. If true, then higher activity levels while housed in the small cages prior to experimental sampling may have resulted in elevated plasma ß-hydroxybutyrate concentrations. Sex was not included in the statistics because there were no females in the Spring group and very few males in the Fall (n=1) and Established (n= 2) groups. It is important to note that the Fall group had fewer animals per flight time than the other two groups and is a between-subject design while the other groups are a within-subject design. This means that the bats that were randomly selected for the longer flight durations in the Fall group might naturally have higher plasma ß-hydroxybutyrate levels or be more active prior to experimental sampling (Figures 2–4).

The changes in resting plasma ß-hydroxybutyrate seen in the Fall group (Figure 2, Figure 3, and Figure 4) could be related to increases in food consumption by the bats when they were not being fasted in captivity and/or due to physiological changes in metabolism that may occur during the fall when bats prepare to overwinter via hibernation. To account for a potential seasonal effect, I collected blood and measured levels of plasma ß-hydroxybutyrate from both rested and exercised fasted bats recently caught from the wild and thus new to captivity in spring 2019. To account for potential

captivity effects, I included the Established group; bats in this group were caught from the wild during May 2016 to May 2018. In the Fall (2018) and Established groups, plasma ß-hydroxybutyrate concentrations increased with increasing durations of physical exertion. Captivity effects are difficult to rule out, as mass changes and regular food consumption can alter basal metabolism (Hochachka, P. W., Darveau, C. A., Andrews, R. D., & Suarez, R. K., 2003). My data indicate that assessment of plasma ß-hydroxybutyrate may provide new insights for assessing activity levels of individual animals, which can inform future field studies.

Future work should explore the rate at which consumed food becomes metabolized into useable fuel in big brown bats. Determining the timeline for the conversion of consumed food into readily available fuel will provide information regarding how long insectivorous bats need to rely on stored lipids at the beginning of the flight foraging period. Future work could also explore what the upper limit of plasma ß-hydroxybutyrate concentration is and how long after starting continuous flight that limit is reached. Further, using carbon isotopes to determine the source of energy (either lipid stores or recently consumed food items) would be useful in developing a timeline for the conversion from consumed food to a useable fuel source, and this would help further our understanding of the physiological mechanisms supporting flight during the early phases of hunting. A direct extension of this project would be to limit food consumption between sample collection so that animals maintain their mass. While increases in mass could be related to seasonal effects or captivity effects, by maintaining mass, it would limit basal metabolic changes. Large mass changes can increase or decrease basal metabolic rates,

which could result in greater ketone body production to supply a larger unmet caloric requirement (Hochachka, 2003).

Mammals go through three phases of fasting which are marked by stereotypical physiological changes (Mellish, J. E., & Iverson, S. J., 200; Castellini, M. A., & Rea, L. D., 1992). Phase one is typically when an animal is in-between meals and is relying on glucogen storage and recently consumed food. Phase two has an increase in ketone body production and an increase in triglyceride levels (Rakvaag et al., 2019; Castellini & Rea, 1992). This is not surprising as acetyl-CoA is a product of ketone body production and a precursor in triglyceride production (Laffel, 1999). Additionally, stored free fatty acids can become trigylcerides through a re-esterification process (Van Ginneken et al., 2007). Phase three starts with an increase in muscle catabolism and results in death (Rakvaag et al., 2019; Castellini & Rea, 1992). It is likely that the bats used by Mcguire et al. (2009*a*) were in phase two of fasting, thus nothing atypical or novel in mammalian physiology was found. However, future studies may wish to explore the fasting phase timeline in big brown bats in more detail to confirm or contradict this result.

Conclusion

The current experiment has advanced the science of mammalian metabolic physiology by addressing a gap in the bat energetics literature. No previous study has measured plasma ß-hydroxybutyrate levels in fasted bats followed by timed exercise (i.e. flight). Fasted bats had higher plasma ß-hydroxybutyrate levels following longer flight times, suggesting that the bats were using fatty acid metabolism to supply energy for flight. Flying while fasted simulates the metabolic conditions that bats are experiencing

during the first emergence from the roost to forage every evening. The results of this experiment support the “fasting while foraging” hypothesis, suggesting that bats are relying on stored fatty acids to supply the energy needed for initially commencing foraging flight.

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Table 1. Description of bat experimental groups to compare when they were sampled from, captivity status, and why they were included in the study.

Group #	Season of Collection	Captivity Status	Reason for Inclusion
1	Fall 2018	Quarantine (New)	Test for seasonal effects. The results were between subjects and not compared statistically to other groups.
2	Spring 2019	Quarantine (New)	Test for seasonal effects, and quarantine status. The results were within subjects and compared between subjects to Group 3.
3	Summer 2019	Established	Test for seasonal effects, and quarantine status. The results were within subjects and compared between subjects to Group 2.

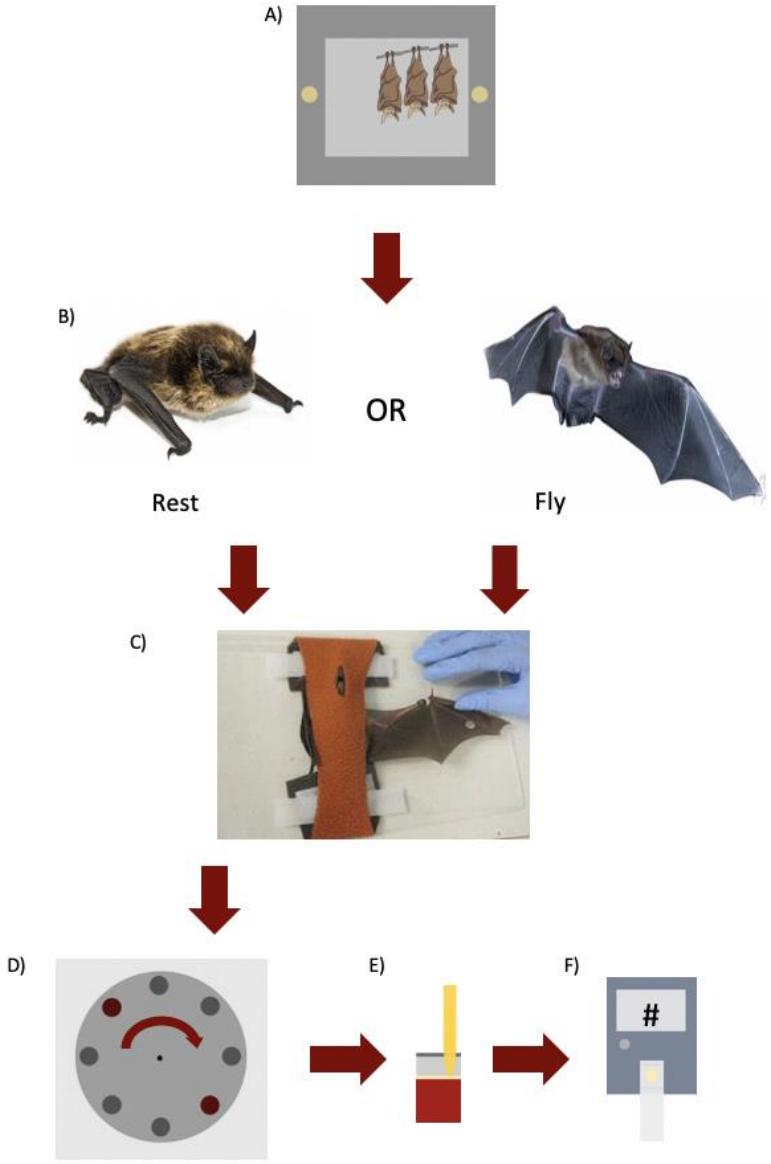

Figure 1. Flow chart depicting the general methods. A) Bats were housed in groups of 3 animals per cage. B) Bats were either flown for varying durations or left at rest. C)Bats were restrained and had blood drawn from the interfemoral vein via a 26-gauge needle. D)Blood was centrifuged at 2000Gs for 10 minutes. E)8 µL of plasma was pipetted onto a test strip. F) STAT-Site M b-HB Photometer (part number 400400; Stanbio Laboratory, Boerne, TX) was used to analyze plasma ß-hydroxybutyrate levels.

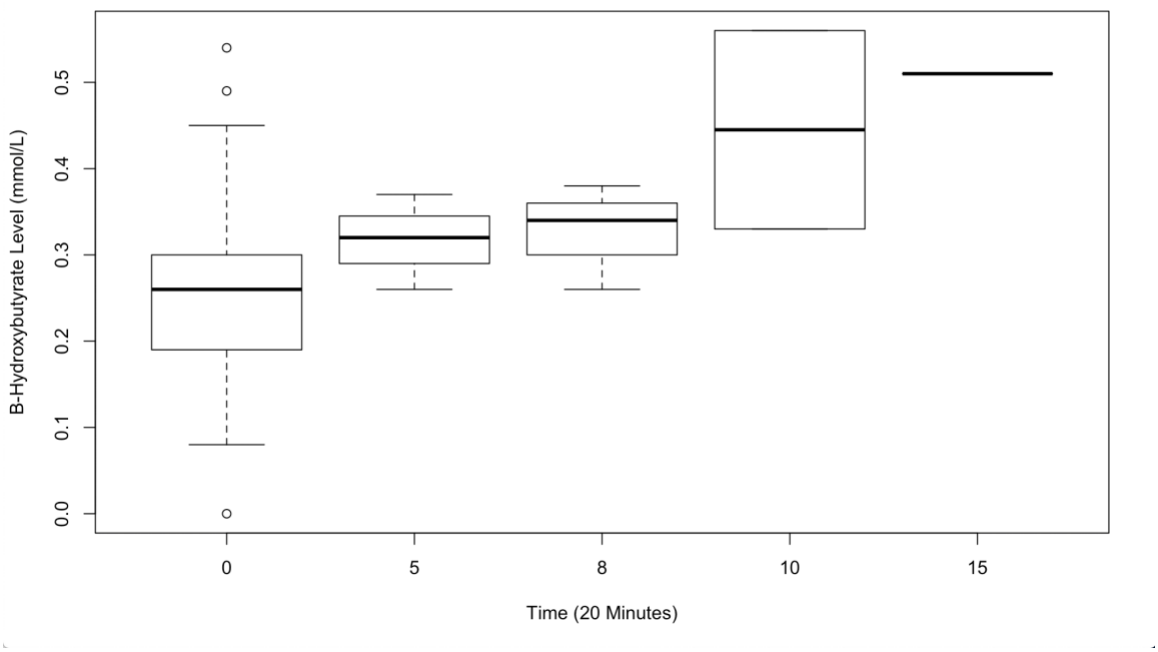

Figure 2. Box and whisker plot of plasma ß-hydroxybutyrate levels after different flight durations – Fall 2018. Plasma ß-hydroxybutyrate levels measured from fasted bats following periods of extended rest or exercise. Data collected for 0, 5, 8, 10, or 15 minutes of exercise reported (n= 26, 3, 3, 3, 1 respectively). Data demonstrate an increase in ß-hydroxybutyrate plasma concentration with increased duration of activity. The bold line in the box represents the mean. The bottom and top of the box represent the 25th and 75th percentiles. The interquartile range (IQR) is the 75th percentile minus the 25th percentile. The bottom and top whiskers represent data points that fall within the minimum (first quartile minus 1.5 times IQR) and the maximum (third quartile plus 1.5 times IQR), respectively; the open circles are data values that fall below and/or above these values.

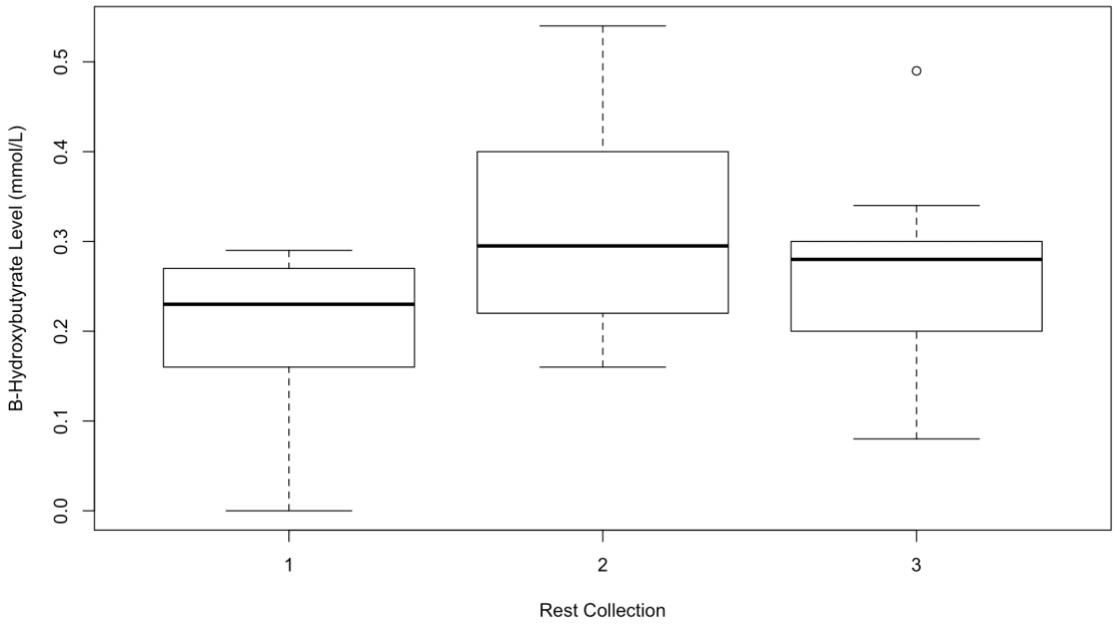

Figure 3. Box and whisker plot of plasma ß-hydroxybutyrate levels after different rest sample collections – Fall 2018. Plasma ß-hydroxybutyrate levels measured from fasted bats following periods of extended rest. Data demonstrate the variability in ß-hydroxybutyrate plasma concentration with rested animals.

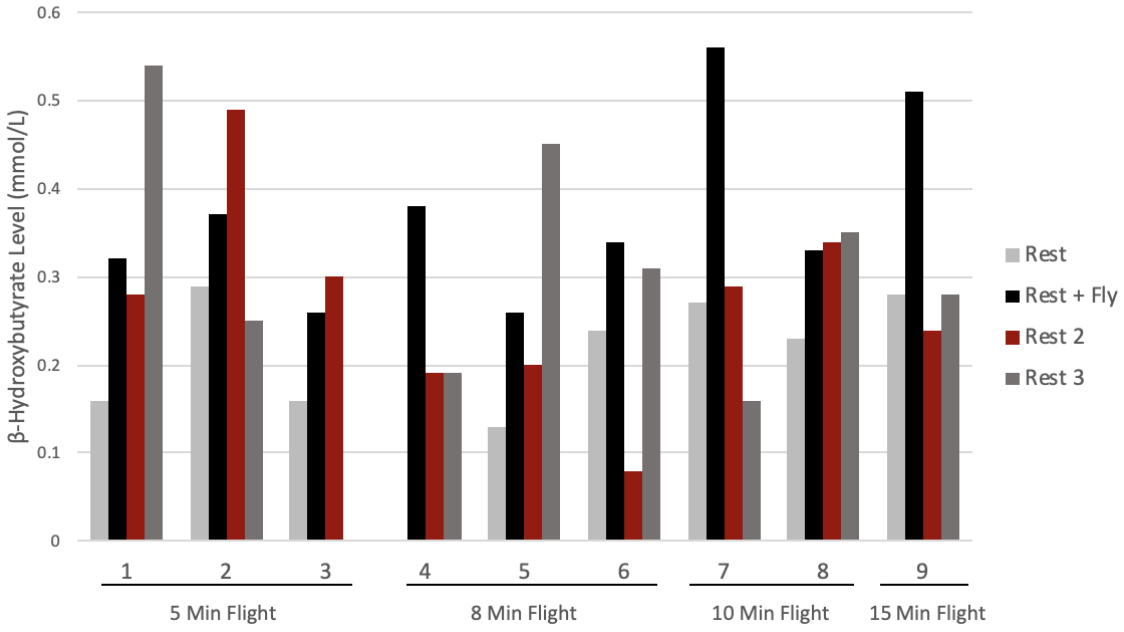

Figure 4. Plasma ß-hydroxybutyrate levels measured from fasted bats following periods of extended rest or exercise – Fall 2018. Data collected for 0, 5, 8, 10, or 15 minutes of exercise reported. Data is grouped together based on the bat the samples were collected from. Bats are labeled 1 through 9. Data demonstrate an increase in ß-hydroxybutyrate plasma concentration with increased duration of activity.

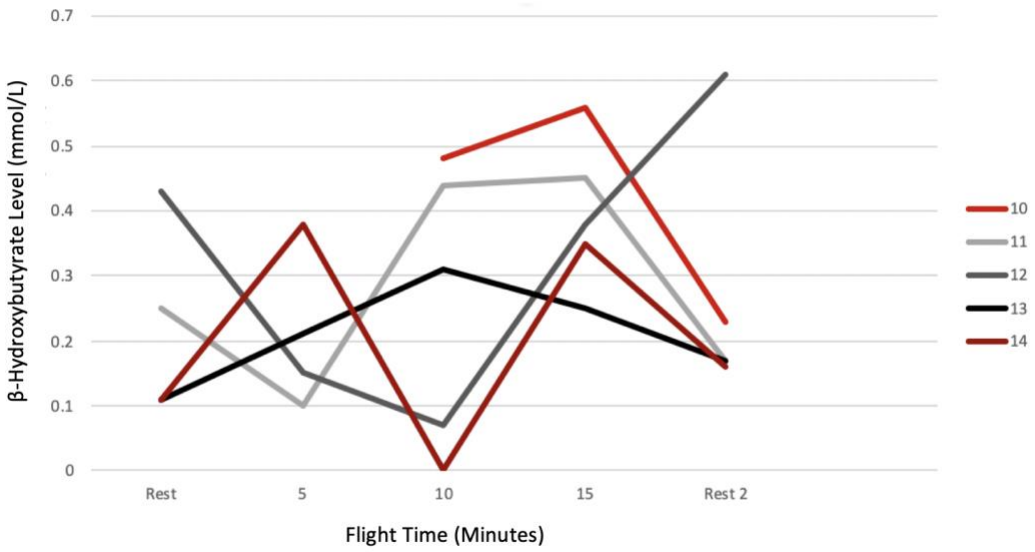

Figure 5. Plasma ß-hydroxybutyrate levels for different flight durations – Spring Group. Plasma ß-hydroxybutyrate levels are recorded at two rest conditions, once following 5 minutes of flight, once following 10 minutes of flight, and once following 15 minutes flight. Observationally, the data show an increase in plasma ß-hydroxybutyrate concentration with increased activity level. The Bat 10 died prior to collecting the 5-min data point.

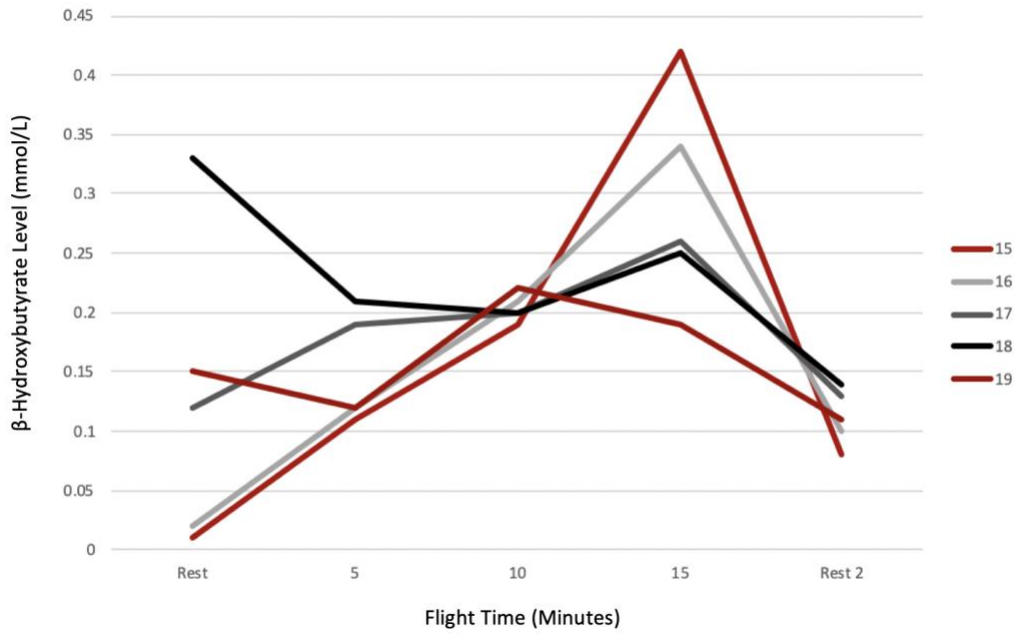

Figure 6. Plasma ß-hydroxybutyrate levels for different flight durations – Established Group. Plasma ß-hydroxybutyrate levels are recorded at two rest conditions, once following 5 minutes of flight, once following 10 minutes of flight, and once following 15 minutes flight. Observationally, the data show an increase in plasma ß-hydroxybutyrate concentration with increased activity level.