ABSTRACT

EFFECTS OF AN IMMUNE RESPONSE ON METABOLISM AND VENTILATION IN BLACK-CAPPED CHICKADEES (POECILE ATRICAPILLUS) DURING COLD STRESS

by

Brian Semanek

Black-capped chickadees (Poecile atricapillius) are small, non-migratory, passerine birds. Birds that are non-migratory that overwinter in cold, temperate regions have increased energetic costs due to thermoregulation during the winter. In addition, food availability may be lower, nighttime fasting is increased, and a decrease in foraging time may make it difficult to acquire the energy required for daily energy demands. If a bird were to develop an infection or acquire a parasite during these winter months, their ability to maintain their body temperature may be affected. The purpose of this study was to observe the effects of an immune response on both the metabolism and ventilation during acute cold exposure in seasonally acclimatized black-capped chickadees.

Chickadees were captured using mist nets in summer months (June-August) and the winter (December). I measured BMR or M_{sum} of chickadees injected with either phosphate buffered saline (PBS) or lipopolysaccharide (LPS). The BMR test used air and kept the chickadees at thermoneutral conditions, while the M_{sum} test used a helox (~79% helium and ~21% oxygen) gas mixture to expose the chickadees to cold stress conditions. Open-circuit respirometry was used to measure oxygen consumption (V02), carbon dioxide production (VCO2), evaporative water loss (EWL) and ventilation.

Thermal conductance, the temperature to become hypothermic or temperature at cold limit (T_{cl}), and evaporative water loss during M_{sum} testing were found to be different between summer and winter caught birds. Metabolic rate and thermal conductance during BMR testing were found to be different between summer and winter caught birds. No difference was found for ventilation measurements in M_{sum} testing. Breathing frequency for BMR testing was different between summer and winter caught birds, while oxygen extraction efficiency for BMR testing was different between phosphate buffered saline and lipopolysaccharide injected birds.

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CHAPTER 1 – INTRODUCTION

Thermoregulation and Energy Metabolism

Energy metabolism has five main components: thermoregulatory metabolism, activity metabolism, basal metabolism, production, and heat increment of feeding or specific dynamic action (Gessman 1987). Thermoregulatory metabolism is metabolism used to counteract heat gain or loss to the environment. Activity metabolism is the metabolism from energy expenditure related to motor activities, such as flying and foraging. Basal metabolism is the minimum amount of energy needed for the maintenance of the organism when the organism is at thermoneutral conditions. Production relates to the energetic costs expended to mate and care for offspring. Finally, heat increment of feeding or specific dynamic action is the energy cost related to digestion and absorption of a meal (Gessman 1987).

Generally, birds are classified as homoeothermic endotherms (Ruben 1995). Homeothermic means that the animal is able to maintain stable internal body temperature, regardless of external influences. An endotherm is an organism that maintains its body at a metabolically favorable temperature largely through the use of heat produced by its internal bodily functions. A narrow temperature range of 38.59 ± 0.96°C when resting to 43.85 ± 0.94°C

when active has been recorded for stable body temperatures in birds (Prizinger et al. 1991). The spinal cord, hypothalamus, and deep-body thermosensors outside of the central nervous system are the primary sites responsible for temperature control in birds (Marsh and Dawson 1989). Shivering thermogenesis is the primary source of heat production in resting birds (Marsh and Dawson 1989). Shivering thermogenesis is a process in which heat is produced through isometric, antagonistic muscle contractions. Flight muscles, such as the pectoralis and supracoracoideus are the main sites of shivering thermogenesis in birds (Bicudo 2001).

The Scholander-Irving model of thermoregulation in endotherms is essential to explain homoeothermic maintenance and avian thermoregulation (Scholander et al. 1950) (Figure 1). The thermoneutral zone (TNZ) is the range of T_as where no heat or cold stress occurs and metabolic rate is relatively constant. At the upper end of the TNZ is the upper critical temperature (T_{uc}) and at the lower end of the TNZ is the lower critical temperature (T_{lc}). Below the T_{lc}, metabolism must increase in response to an increase in heat loss to the surrounding environment. Shivering thermogenesis is the primary method used to raise the body temperature of an organism when the ambient temperature falls below the T_{lc}. Above the T_{uc}, metabolism must increase in response to an increase in heat gain from the surrounding environment. Evaporative cooling techniques, such as gular flutter and panting, are used to

decrease the body temperature of an organism once it has risen above the T_{uc} (Bartholomew et al. 1968).

Metabolic & Ventilatory Rate Measurement

Metabolic rates can be measured using direct or indirect calorimetry. Direct calorimetry uses measurements of energy released as heat over a given period of time to determine metabolic rates (Benzinger and Kitzinger 1949). Usually, the heat released from an animal is transferred to a pre-determined amount of water, and the amount of heat lost from the animal can be calculated based on the increase in temperature of the water (Benzinger and Kitzinger 1949). However, direct calorimetry can be a difficult method to use when attempting to measure low metabolic rates (Randall et al. 2002). Therefore, indirect calorimetry is considered the appropriate method to use in most circumstances. The amount of heat produced in aerobic oxidation is directly related to the amount of oxygen that is consumed through the oxidation of food molecules. Greater than 95% of metabolism in vertebrates is accounted for by aerobic oxidation (Schmidt-Neilson 1997). Therefore, the relationship between the amount of oxygen consumed by oxidation of food molecules (and their products) and the heat produced in aerobic oxidation is significant. Regardless of the substrate being catabolized, the ratio of the amount of heat

produced to the amount of oxygen consumed is almost constant. Therefore, the metabolic rates of organisms can be calculated by measuring the oxygen consumption of that organism. The amount of CO_2 being produced can also be used to calculate the metabolic rate of the organism. If both $V0_2$ and $VC0_2$ are measured in conjunction ($RQ= VCO_2/VO_2$), the individual respiratory quotients (RQ) can be used to estimate total heat production for each metabolic trial using thermal equivalents from Brody (1945).

Open-circuit respirometry is considered to be one of the best methods of measuring metabolic rates of organisms (Gessman 1987; Lighton 2008; Arens and Cooper 2005a) (Figure 2). In open-circuit respirometry, a bird is placed in a metabolic chamber in which air is passed through (Gessman 1987) (Figure 2). Air is pumped through a mass flow meter to achieve a constant air-flow rate into the chamber. The bird meanwhile perches in the air-tight metabolic chamber. The pressure transducer records the ventilation rate by measuring the pressure differences due to the warming and wetting of the air by the breathing bird (Arens and Cooper 2005a; Arens and Cooper 2005b). Air from the chamber is pumped out to a dew point hygrometer, which measures the dew point. Then a sub-sample of the air is passed to the carbon dioxide analyzer to measure carbon dioxide production rate. Finally, all carbon dioxide is removed with a gas scrubber and is passed through an oxygen analyzer that measures the oxygen consumption rate (Gessman 1987).

Effects of Immune Response on Thermoregulation

Several possible competing behavioral and physiological functions require an investment of energy due to condition-dependent life-history traits. Physiological processes such as growth, cellular maintenance, thermoregulation, reproduction, and immune activation require expenditure of energy (Stoehr 2002). Energy resources are limited in an organism and if utilized in one particular process, may limit the effectiveness of another process and thus functionality (Stearns 1989). Both thermoregulation (Swanson 2010) and immune activation (Martin et al. 2003) require high levels of energy expenditure in endothermic organisms. This high amount of energy expenditure can be seen by changes in metabolic output. How organisms distribute the amount of energy between each of these processes is not well documented.

Small passerine birds that remain in a cold climate during winter face high energy expenditures for thermoregulation due to the high surface area to volume ratio of the organism (Schmidt-Nelson 1984; Swanson and Liknes 2005). In winter, birds have reduced foraging time because of a shorter length in days and a lower availability of food because of an increase in snow coverage. Also, winter birds must fast for longer periods during the rest phase compared with summer birds. Adjusting metabolic processes that help

facilitate improved cold tolerance can mitigate some of the negative effects of exposure to the cold (Swanson 2010). Cold tolerance has been defined as the period of time over which a bird can maintain its body temperature by thermogenesis (shivering) at a given level of cold exposure (Swanson 2001). Birds who have become acclimatized to cold temperatures generally show an increase in both summit (Msum, or the maximum metabolic rate during cold exposure) and basal (BMR, or the minimum metabolic rate required for maintenance) metabolic rates compared to birds that have become acclimatized to warmer temperatures (Cooper and Swanson 1994; Arens and Cooper 2005a; Swanson and Liknes 2006). Many winter birds that have increased cold tolerance during cold stress show significant increases in summit metabolism (Swanson 1990; Arens and Swanson 2005b). An increase in Msum is correlated with an increase in cold tolerance in multiple species of birds (Swanson 2001). The energetic costs of an immune response likely have an effect on cold tolerance of birds during exposure to the cold (Burness et al. 2010).

Birds that stay in their habitat over the winter in cold areas must deal with the simultaneous costs of thermoregulation as well as activating and maintaining an immune response if infected with a pathogen (Ots et al. 2001). Activation of the immune system in birds results in an increase in metabolism (Martin et al. 2003; Svensson et al. 1998; King and Swanson 2013; Ots et al.

2001). Birds experiencing the combined effects of an immune challenge and cold exposure have increased resting metabolic rates compared with birds experiencing an immune challenge and are at a thermoneutral temperature (Burness et al. 2010; Hawley et al. 2012). Furthermore, the activation of the immune system has no effect on the thermogenic performance of house sparrows during cold stress (King and Swanson 2013).

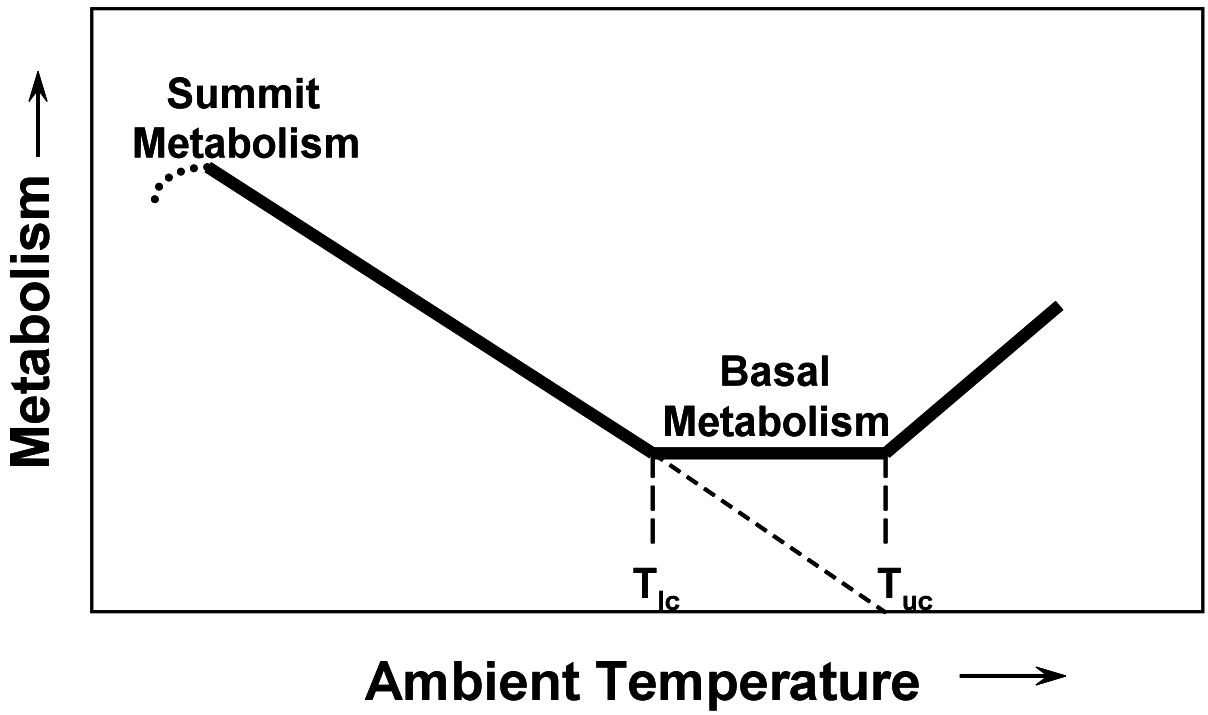

Figure 1. The Scholander-Irving model of thermoregulation.

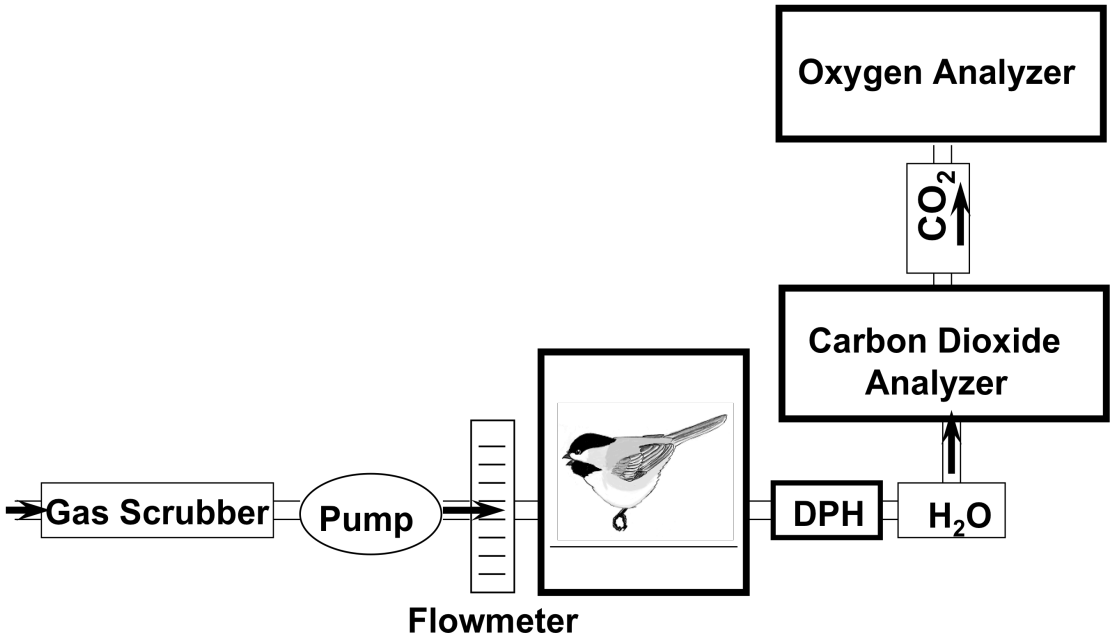

Figure 2. A schematic of the open-circuit respirometry system used in this study. DPH designates the dew point hygrometer. H_20 indicates removal of all H_20. CO_2 indicates removal of all CO_2.

CHAPTER 2 - EFFECTS OF AN IMMUNE RESPONSE ON METABOLISM AND VENTILATION IN BLACK-CAPPED CHICKADEES (POECILE ATRICAPILLUS) DURING COLD STRESS

Introduction

Activation of an immune response in organisms has an energetic cost (Bonneaud et al. 2003; Coon et al. 2011; Hart 1988). Lippopolysaccaride (LPS) was used to produce an acute phase response (APR) in chickadees. LPS simulates a bacterial infection without actually causing the chickadees to become ill. LPS is an immunogenic component of gram-negative bacteria that stimulates a short-lived inflammatory response, but it is not pathogenic (Kent et al. 1992; Klasing et al. 1987; Curtis and Flack 1980). LPS are the major outer surface membrane components present in almost all gram-negative bacteria and are very strong stimulators of innate immune response in most eukaryotic species (Alexander and Rietschel 2001).

The effects of immune activation on the performance and functionality of other condition-dependent life-history traits can be examined though the use of APR (Bonneaud et al. 2003). Behavioral and physiological changes that may affect cold tolerance occur due to the activation of the APR (Owen-Ashley et al and Wingfield 2007). APR in birds leads to the release of endogenous pro-inflammatory cytokines (Kluger et al. 1998), heterothermia (varying body temperature at variable levels), and the presentation of various behaviors

associated typically with sickness (lethargy, adipsia, anorexia, and hypergesia) (Coon et al. 2011; Hart 1988).

Since LPS activates the innate immune system, the effects can be immediately determined and will not be influenced by previous infections. The effects of LPS are costly to an organism and may have an effect on trade-offs between life-history traits (Owen-Ashley and Wingfield 2007). Female house sparrows injected with LPS had decreased nestling feeding rates, reduced success in breeding, and had a decrease in mass when compared with control birds injected with saline (Bonneaud et al. 2003). Zebra finches injected with LPS experienced a decrease in food intake, an increase in nocturnal nesting energy expenditure, and an increase in metabolic expenditure (Burness et al. 2010). Thus, LPS was used in this study as a means of stimulating an immune response to observe energetic costs associated with acute cold exposure. Phosphate-buffered saline (PBS) was used as the control in the study. PBS is water-based, isotonic, and nontoxic to most cells and is a common buffer solution used in biological research.

Black-capped chickadees are small, non-migratory passerine birds. Previous studies determined seasonal variation in metabolism and ventilation (Arens and Cooper 2005a), metabolic and ventilatory acclimatization to cold stress (Arens and Cooper 2005b), the effects of an immune response on metabolism (King and Swanson 2013), and the effects of an injection of

lipopolysaccharide (Owen-Ashley et al. 2005). However, it is not known if there is an effect on ventilation due to an immune response or whether time of year has any determination on the effect of an immune response on metabolism, as previous studies have only examined the effect of an immune response at one particular time of the year (King and Swanson 2013; Owen-Ashley et al. 2005; Bonneaud et al. 2003; Owen-Ashley and Wingfield 2007). Different energetic tradeoffs occur depending on the season. During the summer months, birds need to regulate their body temperature due to heat, reproduce, and there may be a higher chance of infection due to an increased prevalence of parasites (Owen-Ashley and Wingfield 2007). During winter months, birds must significantly regulate their body temperature due to the cold ambient temperatures (Owen-Ashley and Wingfield 2007).

The first goal of this study was to determine if immune activation has an energetic cost, which was determined observing the effects of LPS versus PBS on metabolism in black-capped chickadees during cold stress tests. The second goal of this study was to determine if immune activation affects ventilation, where the effects of LPS versus PBS on ventilation in black-capped chickadees during cold stress tests was observed. The final goal of this study was to determine if there was any seasonal variation in the effects of immune activation. This was determined by whether the time of year the bird was caught, summer versus winter, had any effect on the metabolism and

ventilation of immune activated birds. With these objectives in mind, I predicted that an immune response in chickadees would affect their metabolism and ventilation because an immune response utilizes a portion of the limited resources available to the chickadee. Therefore, birds injected with LPS which undergo a summit metabolic test would have a increase in metabolic rate compared with birds injected with PBS. I also predicted that season will have an influence on the metabolism and ventilation in chickadees due to the increased energetic cost of winter months.

METHODS

Birds.

Black-capped Chickadees (*Poecile atricapillus*) were captured using mist nets between 700 hours and 1000 hours (CST) at Heckrodt Nature Preserve in Menasha, WI and Waukau Creek Nature Preserve, in Winnebago County, from the months of June-August and December 2014. Birds were trapped under state (SRL-NER-131) and federal (MB003340-0) collection permits. Body mass was determined upon capture to the nearest 0.1g using an Ohaus Scout II (Ohaus Corp, NJ) portable electronic balance. Fat scores were visually determined using a 0-5 scale (Helms and Drury 1960), and wing cord and tail lengths were recorded. Age of the chickadees was determined by skull ossifications and breeding characteristics (Pyle et al. 1997). Chickadees were transported back to the laboratory in separate, 30cm x 20cm x 15cm cages, and provided with mealworms, and kept at room temperature during the transportation process. Upon arrival at the laboratory, chickadees were each transferred to separate, 45cm x 28cm x 20cm cages, and were given mealworms, water, sunflower seeds, and grit. All M_{sum} and BMR tests were performed on the day of capture for each chickadee to avoid any effects on metabolism due to captivity (Warkentin and West 1990). After metabolic testing was completed, all Chickadees were banded with a U.S. Fish and Wildlife

Service aluminum leg band (federal banding permit 22934) and released at the original site of capture. All metabolic testing was performed under the University of Wisconsin Oshkosh Institutional Animal Care and Use Committees (protocol number 0026-000274-04-11-14). Chickadees tested between 06/26/2014 and 08/28/2014 were designated summer birds and those tested between 12/09/2014 and 12/22/2015 were designated winter birds.

Injections.

Chickadees were randomly selected to be injected with either LPS or PBS. LPS injections had an injection volume of 0.1 mL and an injection concentration of 0.1 mg / mL. PBS injections had an injection volume of 0.1 mL with no injection concentration (Owen-Ashley et al. 2005). These injection volumes/concentrations are less than other studies that involved larger birds (Martin II et al. 2002), but has been shown to have the same effect on immune response from the time of injection until the end of each test (Owen-Ashley et al. 2005). The injections were performed 3 hours before M_{sum} testing was conducted and 2 hours before BMR testing was conducted to allow for the effects of LPS to begin (the difference in time of injection prior to testing is the first hour of the BMR test not being recorded) (Owen-Ashley et al. 2005; Owen-Ashley and Wingfield 2007, Swanson and Liknes 200). The injection site on the chickadees was below the sternum, into the upper abdomen (Wilson and

Holberton 2002). Traditionally, injections into the pectoral muscle are the most common method. However, attempting an injection into such a small, delicate area on a small bird such as a chickadee has the potential to cause injury. There is no significant difference in an immune response when injecting LPS in the pectoral muscle versus below the sternum (Wilson and Holberton 2002).

Helox Cold Stress.

Maximum cold-induced thermogenesis (M_{sum}) was achieved using 79% helium and 21% oxygen gas mixture (helox). The higher thermal conductivity of helox compared to air facilitates heat loss and allows M_{sum} to be recorded at relatively moderate air temperatures (Rosenmann and Morrison 1974). Helox does not affect measurement of respiratory variable in resting animals, despite the physical differences between helium and nitrogen (Brice and Welch 1983). In addition, helox had no effects on ventilation in house sparrows (*Passer domesticus*) (Arens and Cooper 2005b).

The M_{sum} of individual chickadees was determined by using a sliding cold exposure technique (Swanson et al. 1996). Summer birds were exposed to helox temperatures at a rate of -4°C / 20 min (4°C at the beginning of the test, 0°C twenty minutes into the test, and -4°C forty minutes into the test). Winter birds were exposed to helox temperatures at a rate of -3°C / 20 min (-9°C at the beginning of the test, -12°C twenty minutes into the test, and -15°C forty

minutes into the test. This variable sliding temperature scheme was necessary to cause birds to become hypothermic (indicated during testing by a decrease in oxygen consumption ($V0_2$) over a 3 minute period) at each season in relatively similar times during the test periods. Cold stress tests were conducted between 13:00 and 18:00 (CST). Birds were placed in a 1 L glass metabolic chamber (equipped with a perch over a layer of mineral oil) located inside a Hotpack incubator (model 352602), in which the temperature of the chamber could be controlled within ±0.1°C. Metabolic chamber temperature was continuously recorded (±0.1°C) using a Sable Systems TC1000 thermocouple meter. Chickadees were immediately weighed before and after each test. 15 minutes of equilibration were allowed at the start of each test, followed by exposure to each sliding temperature for a 15-minute period. Individual tests ran for 60 minutes or until the chickadee became hypothermic, indicated by a steady decline in $V0_2$. Once removed from the metabolic chamber, body temperature (T_b) was measure (±0.1°C) with a Cole-Parmer thermocouple thermometer and a 30-gauge copper-constant thermocouple. Body temperature was taken within 30 seconds of removal from the metabolic chamber by inserting the thermocouple into the cloaca at a depth where further insertion did not alter the reading. Body temperature readings of <37°C were considered hypothermic (Swanson et al, 1996).

Summit Metabolic Rate.

Once a chickadee was placed in the metabolic chamber, VO_2 (ml O_2 min^{-1}) was measured during helox cold-stress using open-circuit respirometry. A Cole-Parmer precision rotameter was used upstream of the chamber to regulate flow rate of dry, CO2-free helox at 1341 ml.min^{-1}. Rotameters were calibrated with helox to ±1% accuracy with a soap bubble meter. These flow rates allowed changes in oxygen content between influx and efflux gas of ~0.4-0.8% and maintained efflux oxygen content above 20.1%. A Sable Systems FC-1B oxygen analyzer (Las Vegas, NV) was used to resolve fractional concentration of oxygen in dry, CO_2-free efflux gas. Warthog Systems software (Riverside, CA) and a Sable Systems U12 A/D converter recorded measurements of dry, CO_2-free efflux gas every second. The first fifteen minutes of each test were not included in M_{sum} calculations because they were considered a period of oxygen concentration equilibration. Oxygen consumption rates and carbon dioxide production rates were calculated as instantaneous rates to compensate for the washout characteristics of respirometry systems (Bartholomew et al 1981). Individual respiratory quotients (RQ) were used to estimate total heat production for each metabolic test using thermal equivalents from Brody (1945). M_{sum} data were obtained by averaging VO_2 levels over consecutive 10-minute periods (Dawson and Smith 1986, Swanson 1990). The highest 10-min mean VO_2 was considered to be the

M_{sum} at a given test temperature. Evaporative water loss was calculated using %RH as EWL = FR(F_eH_2O – F_iH_2O) from Warthog Systems Lab Analyzer X. FR is the flow rate ($mL*min^{-1}$), F_eH_2O is the fractional excurrent water vapor density, and F_iH_2O is the fractional incurrent water vapor density (Arens and Cooper 2005b). Warthog System LabHelper X and LabAnalyst X (Riverside, CA) were used to record and analyze VO_2, VCO_2, and EWL measurements, respectively.

Basal Metabolic Rate.

Measurement of basal metabolic rates (BMR) utilized methods similar to the measurement of M_{sum}. However, air was used during BMR testing instead of helox, and two birds were usually tested at the same time. A Sable Systems multiplexer (model TR-RM4) was used to switch the airstream between two individual birds in separate metabolic chambers on 15-minute intervals. All food was removed from the birdcages 4 hours prior to the start of the BMR tests to ensure the birds were in a postabsorptive state. Upon being placed in the chambers, birds were allowed 1 hour of equilibration, followed by 1 hour of recording, where 30 minutes of measurements of each bird were recorded. The two metabolic chambers were checked for gas leaks prior to all tests by momentarily checking efflux gas rates with a flow rotameter. No leaks occurred in either chamber over the course of the tests, as leaks would have been evident by a marked decrease in flow rate compared with the upstream

mass flow meter. Flow rates between 363.7 $ml.min^{-1}$ ± 1% and 388.8 $ml*min^{-1}$ ± 1% for summer birds and between 296.5 $ml.min^{-1}$ ± 1% and 306.9 $ml*min^{-1}$ ± 1% for winter birds were regulated upstream of chambers using two separate omega mass flow controllers (model FMA-A2048). The oxygen analyzer was referenced against incurrent gas prior to and following all tests periods. Chambers were stabilized at a temperature of 30°C±0.1°C for all BMR testing, which is within the thermoneutral zone for chickadees (Cooper and Swanson 1994). BMR testing was conducted between 21:00 and 01:00 (CST) for summer birds and between 18:00 and 23:00 (CST) for winter birds. BMR data are reported as 10-minute minimum means of the last 30 minutes of a trial. Oxygen consumption and CO_2 production were calculated as steady state VO_2 and corrected for STP (Hill, 1972; eq [2]). Evaporative water loss was calculated using %RH as EWL = $FR(F_eH_2O - F_iH_2O)$ from Warthog Systems Lab Analyzer X. Warthog System LabHelper X and LabAnalyst X (Riverside, CA) were used to record and analyze VO_2, VCO_2, and EWL measurements, respectively.

Ventilation Measurements.

Ventilation was measured using whole-body plethysmography in the open-circuit respirometry system (Malan 1973; Bucher 1981). Metabolic chambers had a constant flow rate for all tests. Sable Systems differential

pressure transducers (model PT-100B) were used to measure pressure changes with the chamber due to warming and wetting of inspired air. Data were recorded every 0.05sec for BMR tests and 0.03sec for M_{sum} tests using Warthog LabHelper X software. For BMR tests, chickadees were allowed to equilibrate for 1 hour in the metabolic chambers and then ventilation data were recorded for one hour in each of the two metabolic chambers. For M_{sum} tests, ventilation data were recorded after the 15-minute equilibrium period. 1 mL of air was injected (10-15 times) at the beginning and end of each run to be used for calibration. A known volume of air (1 mL) was injected into the chamber 10-15 times at the beginning and end of each run for calibration. Deflection kinetics during calibration injections was similar to those seen during ventilation (Arens and Cooper 2005b). Calibration and ventilation deflections were compared to compute V_T (Malan 1973 eq. [6]). V_T ($mL*min^{-1}$) and f (min^{-1}) were concurrently with $V0_2$ measurements. The periodicity of ventilation deflections was used to calculate f. Oxygen extraction efficiency (EO_2) was calculated as $EO_2\% = VO_2/(FEO_2 * V_I) * 100$. $FE0_2$ was the fractional oxygen concentration of excurrent air from the metabolic chamber. All ventilation measurements were calculated as both BTPS and STPD (Arens and Cooper 2005b).

Statistics.

Statistical values are presented as means ±SD. BMR with body mass as covariate was not significant. Therefore, all data is presented on a whole organism level. Whole organism data removes possible misleading effects of mass-specific data, which are presented as ratios (Packard and Boardman, 1999). Nested analysis of variance (ANOVA), with classification of season/treatment type as the independent variable, was used to test for significance between the groups for T_a, Tb_{out}, Watts, thermal conductance, evaporative water loss, breathing frequency, minute volume, tidal volume, and oxygen extraction efficiency. This ANOVA was performed on the data from the BMR tests and again with the data from the M_{sum} tests. A nested ANOVA was also performed on the temperature at the start of the experiment for M_{sum} data and the BMR data. A nested ANOVA was also performed on the difference in temperature at the time of injection and the start of the experiment for M_{sum} data and the BMR data. Tukey's post hoc multiple comparisons tests were performed along with all ANOVAs. Repeated measures ANOVA was conducted on the different temperature measurements starting at the time of injection and ending hours after the respected test was concluded. A separate repeated measures test was conducted for M_{sum} winter, M_{sum} summer, BMR winter, and BMR summer. Paired t-tests were conducted to determine if treatment (PBS/LPS) caused a difference in means between the body temperature at the

time of injection and at the start of the metabolic test. Likewise, paired t-tests were conducted to determine if treatment (PBS/LPS) caused a difference in means between the mass at the time of injection and the body temperature at the start of the metabolic test . The calculated t and P-values were recorded. All statistics were computed using SPSS 13.0 (SPSS, Chicago). Statistical significance was accepted at $P<0.05$.

RESULTS

Basal Metabolic Rate.

Not all black-capped chickadees displayed hypothermia during BMR testing. PBS-injected winter birds (2 of 7 birds), LPS-injected summer birds (5 of 7 birds), PBS-injected winter birds (2 of 6 birds), and LPS-injected winter birds (2 of 6 birds) became hypothermic before being removed from the metabolic chamber. Many different species of similar birds, such as common redpolls (*Acanthis flammea*), willow tits (*Poecile montanus*), and great tits (*Parus major*), exhibit a relationship between the degree of hypothermia the bird experiences and the amount of energy reserves available to the bird (Reinertsen and Haftorn 1983, 1986).

Changes in seasonal cold tolerance could be affected by seasonal changes in thermal conductance. Thermal conductance ($C = M_{sum} / [T_b - T_a]$; Sholander et al., 1950) was therefore calculated and compared between seasons and immune treatments. Thermal conductance was different for season ($F_{1,20}$=21.284, P<.050). For drug treatment type nested within season, no difference in thermal conductance was found ($F_{2,20}$=0.800, P=0.463) (Table 2).

BMR (watts) was different between seasons ($F_{1,20}$=57.888, P<0.05). but not between drug treatment type nested within season ($F_{2,20}$=0.463, P=0.636). Thus, season had an effect on BMR (watts), while drug treatment type nested within season did not (Table 2).

Evaporative water loss was not different between seasons ($F_{1,20}$=10.863, P=0.081). Evaporative water loss was also not different for drug treatment type nested within season ($F_{2,20}$=0.363, P=0.700) (Table 2).

Summit Metabolic Rate.

All black-capped chickadees displayed hypothermia during M_{sum} testing (seven PBS-injected summer birds, seven LPS-injected summer birds, six PBS-injected winter birds, and six LPS-injected winter birds became hypothermic before being removed from the metabolic chamber).

The temperature at cold limit (T_{cl}) was different for season ($F_{1,21}$=43.496, P<0.05), but not for drug treatment type nested within season ($F_{2,21}$=2.732, P=0.688). Therefore, season (summer/winter) had an effect on T_{cl}, while drug treatment type (PBS/LPS) did not (Table 1).

Changes in seasonal cold tolerance could be affected by seasonal changes in thermal conductance. Thermal conductance ($C = M_{sum} / [T_b - T_a]$; Sholander et al., 1950) was therefore calculated and compared between seasons and immune treatments. Thermal conductance was different for season ($F_{1,21}$=42.496, P<0.05), but not for drug treatment type nested within season ($F_{2,21}$=0.381, P=0.688). Therefore, season (summer/winter) has a significant effect on thermal conductance, while drug treatment type (PBS/LPS) did not (Table 1).

M_{sum} (watts) was to not be different between seasons ($F_{1,21}$=16.395, P=0.056). Drug treatment type nested within season was also not different ($F_{2,21}$=0.873, P=0.433) (Table 1). Thus, neither season (summer/winter) nor drug treatment type (PBS/LPS) nested within season had any effect on the summit metabolic rate.

Evaporative water loss was different for season ($F_{1,21}$=479.526, P<0.001), but not for drug treatment type nested within season ($F_{2,21}$=0.022, P=0.978). Therefore, season (summer/winter) had an effect on evaporative water loss, while drug treatment type (PBS/LPS) nested within season did not (Table 1).

Ventilation.

For M_{sum} testing, breathing frequency was not different due to season ($F_{1,21}$=14.219, P=0.056) or drug treatment type nested within season (F2,21=0.035, P=0.965) (Table 3). For BMR testing, breathing frequency was different due to season ($F_{1,20}$=22,518, P<0.05), while drug treatment type nested within season was not different ($F_{2,20}$=1.120, P=0.346) (Table 4).

Tidal volume at standard pressure and density from Msum testing was not different due to season ($F_{1,21}$=0.199, P=0.699) or drug treatment type nested within season ($F_{2,21}$=1.059, P=0.365) (Table 3). Similarly, tidal volume at standard pressure and density from BMR testing was not different for either

season ($F_{1,20}$=0.076, P=0.809) or drug treatment type nested within season ($F_{2,20}$=1.679, P=0.212) (Table 4).

Minute volume at standard pressure and density from Msum testing was not different due to season ($F_{1,21}$=0.051, P=0.843) or drug treatment type nested within season ($F_{2,21}$=0.595, P=0.561) (Table 3). Similarly, minute volume at standard pressure and density from BMR testing was not different for either season ($F_{1,20}$=8.220, P=0.103) or drug treatment type nested within season ($F_{2,20}$=2.141, P=0.144) (Table 4).

Oxygen extraction efficiency at standard pressure and density from Msum testing was not significantly different due to season ($F_{1,21}$=0.029, P=0.881) or drug treatment type nested within season ($F_{2,21}$=0.783, P=0.470) (Table 3). Minute volume at standard pressure and density from BMR testing was not different for season ($F_{1,20}$=2.849, P=0.233) but was different for drug treatment type nested within season ($F_{2,20}$=3.562, P<0.05) (Table 4).

Body Temperature.

Four repeated measure tests were conducted to determine if there was a difference in temperature due to either time after injection or treatment type: M_{sum} summer birds, M_{sum} winter birds, BMR summer birds, and BMR winter birds (Table 5 and Table 6). Within-subject factors were the various times when the temperature of the bird was taken after injection. The between-

subject factors were the treatment type: LPS or PBS injection. For M_{sum} summer, there was no difference in between-subject effects (F_1=0.105, P=0.752), for M_{sum} winter, there was no difference in between-subject effects (F_1=0.008, P=0.930), for BMR summer, there was a difference in between-subject effects (F_1=7.181, P<0.05) and for BMR winter, there was no difference in between-subject effects (F_1=0.939, P=0.355).

To determine if time had a significant effect on body temperature, one-way ANOVA was performed for all M_{sum} data (PBS summer, PBS winter, LPS summer, PBS winter) and another for all BMR data (PBS summer, PBS winter, LPS summer, PBS winter) comparing T_b at the start of each experiment. There was no difference for either M_{sum} ($F_{3,22}$=2.600, P=0.078) or BMR ($F_{3,22}$=2.816, P=0.063). However, the times after the experiment, when any immune response from LPS would be wearing off, may have skewed the results. Another one-way ANOVA was performed using all data from both sets of tests together to determine if there was any difference in $T_{b\ inject} - T_{b\ start}$. The one-way ANOVA showed that there was no difference (F3,48=1.939, P=0.136).

Paired t-tests were conducted to determine if there was a difference in means of the body temperature at the time of injection compared to the time the metabolic test began ($\Delta T_{b)}$. T-tests did not distinguish BMR-tested birds from M_{sum}-tested birds because the two, recorded temperatures were obtained prior to testing. Therefore, paired t-tests were conducted for: PBS-injected

summer birds, LPS-injected birds summer, PBS-injected winter birds, and LPS-injected winter birds. For PBS-injected summer birds mean ΔT_b differed (t_{13}=2.275, P<0.05), for LPS-injected summer birds mean ΔT_b also differed (t_{13}=8.387, P<0.001), and a difference of mean ΔT_b was found for PBS injected winter birds (t_{13}=3.438, P<0.005). However, there was no difference in mean ΔT_b of LPS-injected winter birds (t_{13}=2.037, P=0.063).

Body Mass.

Paired t-tests were conducted to determine if there was a significant difference in means of the mass at the time of injection compared to the mass at the time the metabolic test began. T-tests did not distinguish BMR-tested birds from M_{sum}-tested birds because the two recorded masses were obtained prior to any testing. Therefore, four paired t-tests were conducted, as in the tests for body temperature (Table 7 and Table 8). For summer, PBS-injected birds, means differed (t_{15}=3.467, P<0.005), for summer, LPS-injected birds, means differed (t_{15}=4.105, P<0.005), and means differed for winter, LPS-injected birds (t_{11}=2.572, P=0.026). However, no difference of means was found for winter PBS-injected birds (t_{11}=0.000, P=1.000).

DISCUSSION

Basal Metabolic Rate.

PBS-injected winter chickadees had a higher mean BMR than PBS-injected summer chickadees or LPS-injected summer chickadees (Table 2, Figure 4). Likewise, LPS-injected winter chickadees had a higher mean BMR than PBS-injected summer chickadees or LPS-injected summer chickadees (Table 2, Figure 4). This indicates that treatment type (PBS or LPS) had no effect on the mean BMR of the chickadees, but season (summer or winter) did have an effect. These variations in basal metabolic rates due to season are consistent with other species of birds (Liknes et al. 2002, Cooper 2002, Arens and Cooper 2005b). Swanson (1991) suggested that the increased metabolic costs of thermogenesis in winter might cause the increase in metabolic rate. It may also be due to the increased mass of pectoralis muscles. The increased shivering thermogenesis in winter corresponds to an increase in pectoralis mass. Many species of birds, such as mountain chickadees (*Poecile gambeli*), house finches (*Haemorhous mexicanus*), juniper titmice (*Baeolophus ridgwayi*), and dark-eyed juncos (*Junco hyemalis*), have increased pectoralis muscle mass during the winter (O'Connor 1995; Cooper 2002; Swanson 1991).

PBS-injected summer chickadees had a lower mean thermal conductance than either PBS-injected winter chickadees or LPS-injected winter chickadees (Table 2, Figure 4). This shows that treatment type (PBS or LPS) has no effect

on mean thermal conductance in chickadees, but season (summer or winter) does have an effect. These results are similar to those of other studies of passerine birds that show the importance of insulatory adjustments to seasonal acclimatization (Liknes et al. 2002; Swanson 1991). Small passerine birds acclimatize to winter conditions through metabolic adjustments (Marsh and Dawson 1989). These metabolic adjustments lead to higher thermogenic endurance (Swanson 1990; Cooper and Swanson 2004) and cold tolerance (Barnett 1970) in winter birds compared to summer birds.

Evaporative water loss was found to not be different between seasons and was also found to be not different for drug treatment type nested within season (Table 2).

Summit Metabolic Rate.

PBS-injected winter chickadees did not have a higher mean M_{sum} than PBS-injected summer and LPS-injected summer chickadees (Table 1, Figure 3). This indicates that treatment type (PBS/LPS) and season (summer/winter) had no effect on the mean M_{sum} of the chickadees. These findings are in contrast to those previously of a higher M_{sum} in winter birds than in summer birds (Liknes and Swanson 1996, Cooper and Swanson 1994, Cooper 2002). Recorded M_{sum} values for other small, passerine birds caught during the winter have an increase of 0% to 52% compared to the same species of birds caught during the

summer (Cooper and Swanson 1994; Liknes and Swanson 1996). These differing results may be due to an abnormally cold winter occurring previous to the summer which chickadees were caught causing a change in seasonal acclimatization (Arens and Cooper 2005, Swanson). Also, the winter in which chickadees were caught was a very mild winter. The temperatures of the previous 14 days prior to capture can have a significant effect on metabolic rates of birds (Swanson and Olmstead 1999). Thus, the metabolic rates of the captured chickadees may have been affected by the unusually mild previous days prior to capture.

There was also difference in the mean temperature to become hypothermic. PBS-injected summer chickadees had a lower mean T_{cl} than either PBS-injected winter chickadees or LPS-injected winter chickadees (Table 1). LPS-injected summer chickadees also had a lower mean T_{cl} than either PBS-injected winter chickadees or LPS-injected winter chickadees (Table 1, Figure 3). Therefore, season (summer or winter) has an effect on mean T_{cl} in chickadees, but treatment (PBS or LPS) does not have an effect. This increase in T_{cl} during the winter normally coincides with an increase in M_{sum} during the winter, suggesting there may be a correlation between the two. This correlation has been supported by several other studies (Arens and Cooper 2005b, Swanson 2001). However, there was no significant difference in M_{sum} due to season in this study, indicating there is no correlation between M_{sum} and

T_{cl}. The physiological adjustments that produce increased thermogenic endurance should also produce an increased maximum cold-induced metabolic rate in small birds (Swanson 2001). As previously noted, the warmer than usual proximate temperatures prior to the capture of chickadees during the winter may be the cause of this discrepancy (Swanson and Olmstead 1999).

LPS-injected winter chickadees had higher mean thermal conductance than either PBS-injected summer or LPS-injected summer chickadees (Table 1, Figure 3). This shows that treatment type (PBS or LPS) has no effect on mean thermal conductance in chickadees, but season (summer or winter) does have an effect. These results are similar to those found in other studies of passerine birds (Arens and Cooper 2005a; Swanson and Liknes 2005; Swanson 1991). Small passerine birds acclimatize to winter conditions through metabolic adjustments (Marsh and Dawson 1989). These metabolic adjustments lead to higher thermogenic endurance (Swanson 1990; Cooper and Swanson 2004) and cold tolerance (Barnett 1970) in winter birds compared to summer birds.

There was a difference in the mean evaporative water loss in chickadees during cold stress testing. PBS-injected summer chickadees had a higher mean evaporative water loss than either PBS-injected winter chickadees or LPS-injected winter chickadees (Table 1, Figure 3). Likewise, LPS-injected summer chickadees had a higher mean evaporative water loss than either PBS-injected winter chickadees or LPS-injected winter chickadees (Table 1, Figure 3).

Therefore, season (summer or winter) has an effect on mean evaporative water loss in chickadees, but treatment (PBS or LPS) does not have an effect.

Ventilation.

There was no difference in mean breathing frequency from M_{sum} testing (Table 3, Figure 5). This is in contrast to a previous study with house sparrows (*Passer domesticus)*, where breathing frequency during cold stress was different due to season (Arens and Cooper 2005b). However, there was a difference in mean breathing frequency from BMR-testing. PBS-injected summer chickadees had a lower mean breathing frequency than either PBS-injected winter chickadees or LPS-injected winter chickadees (Table 4, Figure 7). LPS-injected summer chickadees also had a lower mean breathing frequency than either PBS-injected winter chickadees or LPS-injected winter chickadees (Table 4, Figure 7). Therefore, season has an effect on mean breathing frequency during BMR tests, but treatment type did not. Breathing frequency at thermoneutral conditions has been found to be different due to season in house sparrows (*Passer domesticus*) in previous studies as well (Arens and Cooper 2005b).

There was no difference in mean tidal volume STPD (Temperature of 0°C and a pressure of 760 mm Hg) from either M_{sum}-testing or BMR-testing (Table 3 and Table 4, Figure 6 and 8). These are the same results found in a previous

study involving house sparrows (*Passer domesticus*) (Arens and Cooper 2005b). There was no difference in mean minute volume STPD from M_{sum}-testing (Table 3, Figure 6). However, there was a difference in mean minute volume STPD from BMR-testing. PBS-injected summer chickadees had a lower mean minute volume STPD than either PBS-injected winter chickadees or LPS-injected winter chickadees (Table 4, Figure 8). Both mean minute volume STPD from M_{sum}-testing and BMR-testing was found to be different due to season in a previous study (Arens and Cooper 2005b). Therefore during BMR-testing, season had an effect on mean minute volume STPD, while treatment did not.

There was no difference in mean oxygen extraction efficiency STPD for M_{sum}-testing (Table 3, Figure 5). However, there was a difference in mean oxygen extraction efficiency STPD for BMR-testing. PBS-injected summer birds had higher mean oxygen extraction efficiency STPD than either LPS-injected summer birds or LPS-injected winter birds (Table 4, Figure 7). These results of season having no effect match those of a previous study involving house sparrows (*Passerus domesticus*), which showed that there was no difference in mean oxygen extraction efficiency due to season from either BMR-testing or M_{sum}-testing (Arens and Cooper 2005b). Thus, season had no effect on mean oxygen extraction efficiency STPD, but treatment did. Why treatment affected mean oxygen extraction efficiency, but no other ventilatory or metabolic measurements is unknown.

There were no differences in any ventilation measurements for M_{sum}-testing (Table 3, Figure 5 and Figure 6). BMR-testing had differences in breathing frequency due to season and minute volume STPD due to treatment (Table 4, Figure 7 and Figure 8). This difference in mean breathing frequency may be due to seasonal acclimatization in birds (Arens and Cooper 2005b). However, variations in ventilation measurements have only been found in black-capped chickadees and downy woodpeckers and are not a universal occurrence (Cooper, published abstract). An increase in mean breathing frequency from BMR-testing during the winter coincides with the increase in basal metabolic rate from BMR-testing during the winter found in this study. This correlation between breathing frequency and basal metabolic rate has been found in the previous study with house sparrows (*Passerus domesticus*) (Arens and Cooper 2005b). There were also differences in mean oxygen extraction efficiency STPD due to treatment type in BMR-testing, but the cause for could not be determined.

Body Temperature.

Paired t-tests were conducted to determine if there was a difference in mean body temperature at the time of injection compared to the time the metabolic test began (ΔT_b). Of the four, paired t-tests completed, three of the four revealed a difference of mean ΔT_b: summer PBS-injected birds, summer

LPS-injected birds, and winter PBS-injected birds (Table 5, Table 6, and Figure 9). These results are inconclusive as to the effects of season or injection type because both PBS and LPS injected birds showed significant differences of mean ΔT_b. Likewise, winter and summer birds both showed a difference in mean ΔT_b. These results are in contrast with those found previously, whereby LPS decreased ΔT_b (King and Swanson 2013; Owen-Ashley et al. 2005). However, there have been no previous studies to test the effect season plays on temperature change due to an immune response.

Body Mass.

Paired t-tests were conducted to determine if there was a significant difference in means of the mass at the time of injection compared to the mass at the time the metabolic test began (ΔM). Of the four, paired t-tests completed, three had different mean ΔM: Summer PBS-injected birds, summer LPS-injected birds, and winter LPS-injected birds (Table 7, Table 8, and Figure 10). No trend can be identified from these results because both PBS and LPS showed differences of mean ΔM. Likewise, winter and summer birds both showed a difference in mean ΔM. These results are similar to those found previously with sparrows (*Passeridae*), where there was no difference in ΔM found between birds injected with PBS and those injected with LPS (King and

Swanson 2013; Owen-Ashley et al. 2005) and season has no effect on mean ΔM in sparrows (*Passeridae*) (Martin II et al. 2003).

Final Conclusions.

Throughout the course of this study, an immune response, due to the effects of injection with LPS, did not result in any differences in mean metabolic rate, thermal conductance, temperature at cold limit, and evaporative water loss for either BMR-testing or M_{sum}-testing. Likewise, an immune response had no effect on breathing frequency, tidal volume, minute volume, or oxygen extraction efficiency for either BMR-testing or M_{sum}-testing (except oxygen extraction efficiency from BMR-testing). Mean body temperature and mean body mass from both BMR-testing and Msum-testing were not different due injection of LPS as well. Therefore, an immune response does not have an effect on any metabolic or ventilatory measurements in black-capped chickadees (*Poecile atricapillius*) (other than EO_2 for BMR-testing).

Season had a significant effect on both metabolic and ventilatory measurements for both BMR-testing and M_{sum}-testing. Mean thermal conductance and mean evaporative water loss were different due to season for M_{sum}-testing and basal metabolic rate, thermal conductance and mean breathing frequency were different due to season for BMR-testing. Thus,

season has a significant effect on both metabolic and ventilatory measurements in black-capped chickadees (*Poecile atricapillius*).

Table 1. *Mean (±SD) values for conductance, evaporative water loss, and metabolic rate for birds during M_{sum} testing*

Season / Treatment	Conductance ($W*°C^{-1}$)	EWL (ml/min)	Metabolic Rate (Watts)	T_{cl} (°C)
Summer:				
PBS	8.042±1.766*	2.954±0.588*	1.124±0.033	20.814±0.212*
LPS	7.658±0.943*	2.979±1.262*	1.136±0.070	20.600±0.141*
Winter:				
PBS	11.530±2.787*	1.831±0.575*	1.379±0.182	23.033±0.450*
LPS	12.819±4.209*	1.735±0.553*	1.280±0.182	23.233±1.178*

* Indicates significant difference between seasons ($P<0.05$)

Table 2. Mean (±SD) values for conductance, evaporative water loss, and metabolic rate for birds during BMR testing

Season / Treatment	Conductance ($W*°C^{-1}$)	EWL (ml/min)	Metabolic Rate (Watts)
Summer:			
PBS	1.069±0.063*	1.230±0.440	0.190±0.019*
LPS	1.223±0.080*	1.048±0.343	0.198±0.010*
Winter:			
PBS	1.492±0.332*	0.837±0.427	0.230±0.028*
LPS	1.525±0.254*	0.711±0.564	0.235±0.011*

* Indicates significant difference between seasons ($P<0.05$)

Table 3. Mean (±SD) ventilation values of chickadees in summer and winter during M_{sum} testing

Season / Treatment	Breathing Freq (Hz)	V_i - STPD (ml 0_2)	V_T-STPD (ml 0_2*S^{-1})	E02 (STPD) (%)
Summer:				
PBS	196.487±33.434	175.267±38.642	0.897±0.144	13.946±4.375
LPS	192.442±30.127	154.142±54.383	0.788±0.202	15.086±3.260
Winter:				
PBS	206.195±31.787	182.126±65.713	0.868±0.212	13.077±2.484
LPS	202.888±42.904	154.988±61.812	0.744±0.238	16.583±8.139

* Indicates significant difference between seasons (P<0.05)

Table 4. Mean (±SD) ventilation values of chickadees in summer and winter during BMR testing

Season / Treatment	Breathing Freq (Hz)	V_i - STPD (ml 0_2)	V_T-STPD (ml 0_2*S^{-1})	E02 (STPD) (%)
Summer:				
PBS	45.545±1.703*	10.038±2.941	0.220±0.061	28.108±6.780**
LPS	56.516±14.248*	15.442±3.907	0.278±0.073	19.530±7.643**
Winter:				
PBS	96.145±33.606*	20.048±5.494	0.216±0.041	16.985±3.007**
LPS	83.908±10.582*	21.411±5.739	0.262±0.096	16.097±3.445**

* Indicates significant difference between seasons (P<0.05)
** Indicates significant differences between treatment (P<0.05)

Table 5. Mean T_bs (°C) for birds undergoing M_{sum}-testing

Season / Treatment	Time since injection (hours)	T_b (°C)
Summer:		
PBS	0	39.9
PBS	3	39.6
PBS	4	34.0
PBS	6	40.1
PBS	18	40.4
LPS	0	40.0
LPS	3	38.4
LPS	4	34.6
LPS	6	40.2
LPS	18	40.5
Winter:		
PBS	0	39.9
PBS	3	39.1
PBS	4	33.8
PBS	6	39.0
PBS	18	38.1
LPS	0	39.4
LPS	3	39.6
LPS	4	33.2
LPS	6	39.4
LPS	18	38.0

Table 6. Mean T_bs (°C) for birds undergoing BMR-testing

Season / Treatment	Time since injection (hours)	T_b (°C)
Summer:		
PBS	0	40.1
PBS	3	38.4
PBS	5	37.4
PBS	12	40.1
LPS	0	39.1
LPS	3	37.1
LPS	5	36.1
LPS	12	39.7
Winter:		
PBS	0	39.7
PBS	3	38.6
PBS	5	37.4
PBS	12	39.4
LPS	0	39.5
LPS	3	37.9
LPS	5	37
LPS	12	39.3

Table 7. Mean mass (g) for birds undergoing M_{sum}-testing

Season / Treatment	Time since injection (hours)	Mass (g)
Summer:		
PBS	0	11.2
PBS	3	11.1
PBS	4	10.8
PBS	6	10.8
PBS	18	10.8
LPS	0	11.2
LPS	3	10.9
LPS	4	10.7
LPS	6	11.0
LPS	18	10.7
Winter:		
PBS	0	11.6
PBS	3	11.8
PBS	4	11.5
PBS	6	11.9
PBS	18	11.1
LPS	0	11.2
LPS	3	11.1
LPS	4	10.9
LPS	6	11.4
LPS	18	10.7

Table 8. Mean mass (g) for birds undergoing BMR-testing

Season / Treatment	Time since injection (hours)	Mass (g)
Summer:		
PBS	0	10.9
PBS	3	10.7
PBS	5	10.5
PBS	12	10.4
LPS	0	11.3
LPS	3	11.1
LPS	5	10.9
LPS	12	10.8
Winter:		
PBS	0	11.4
PBS	3	11.1
PBS	5	10.9
PBS	12	10.7
LPS	0	11.4
LPS	3	11.0
LPS	5	10.8
LPS	12	10.4

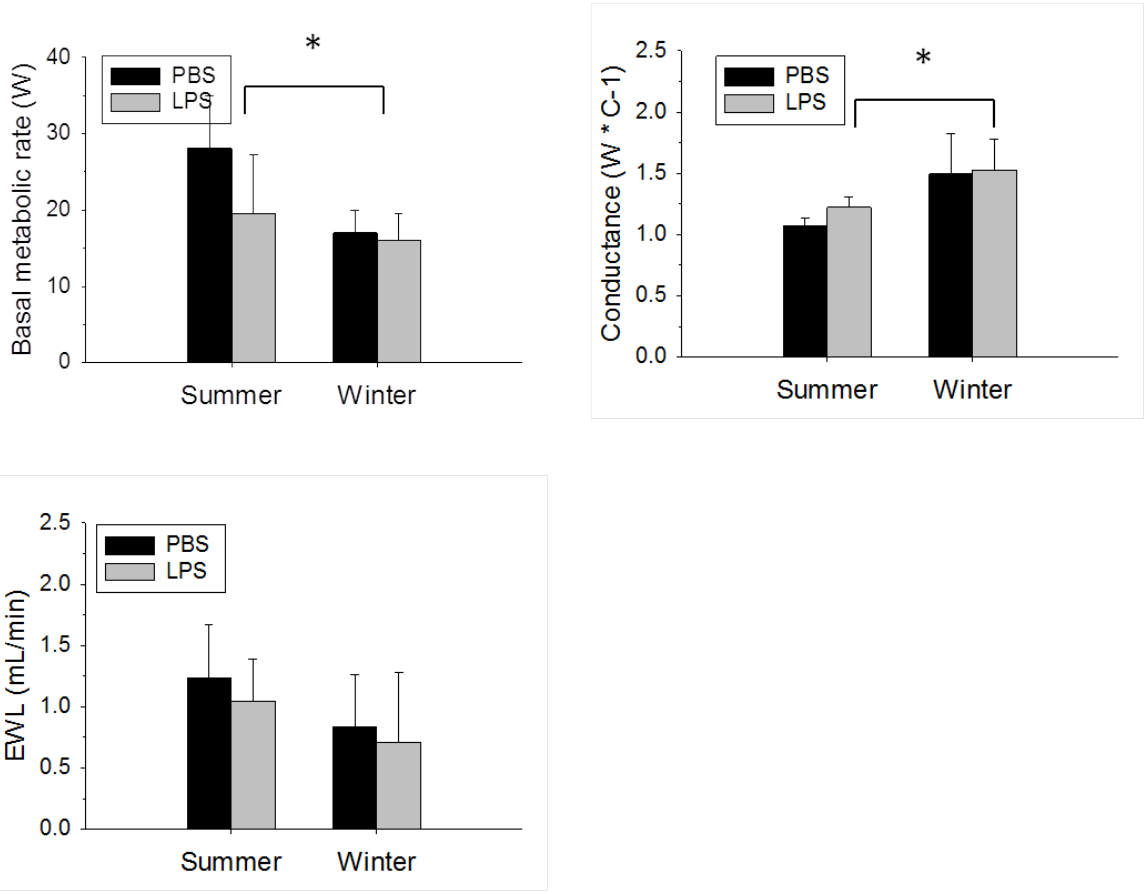

Figure 3. Mean summit metabolic rate, mean thermal conductance, and mean evaporative water loss (EWL) during helox cold stress in summer and winter black-capped chickadees injected with either PBS or LPS.

* Indicates significant difference between seasons ($P<0.05$)

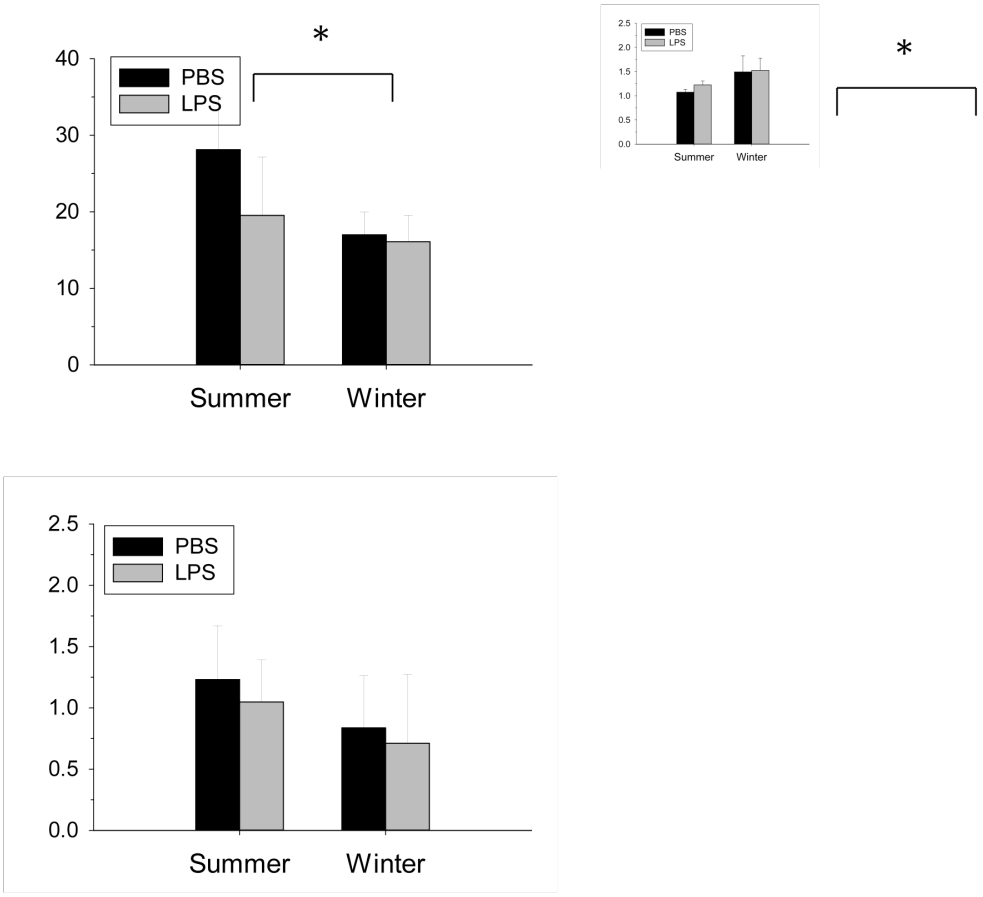

Figure 4. Mean basal metabolic rate, mean thermal conductance, and mean evaporative water loss (EWL) at thermoneutral conditions in summer and winter black-capped chickadees injected with either PBS or LPS.

* Indicates significant difference between seasons ($P<0.05$)

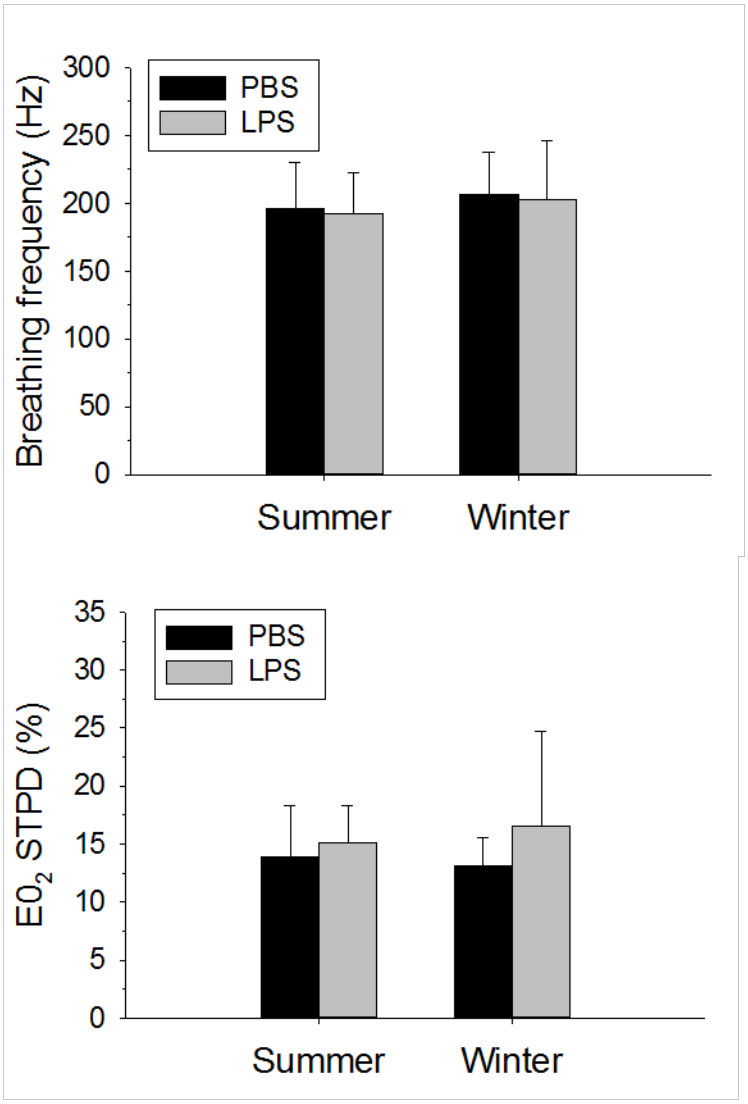

Figure 5. Mean breathing frequency and oxygen extraction efficiency (EO_2) during helox cold stress in summer and winter black-capped chickadees injected with either PBS or LPS.

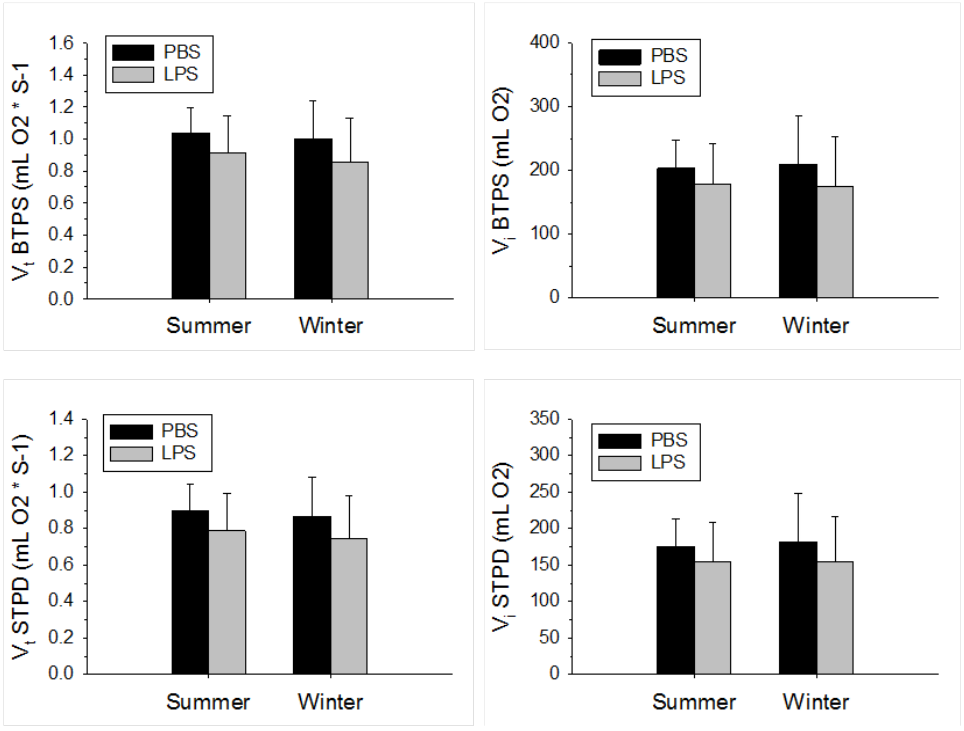

Figure 6. Mean tidal volume (V_t) BTPS, mean tidal volume (V_t) STPD, mean minute volume (V_i) BTPS, and mean minute volume (V_i) STPD during helox cold stress in summer and winter black-capped chickadees injected with either PBS or LPS.

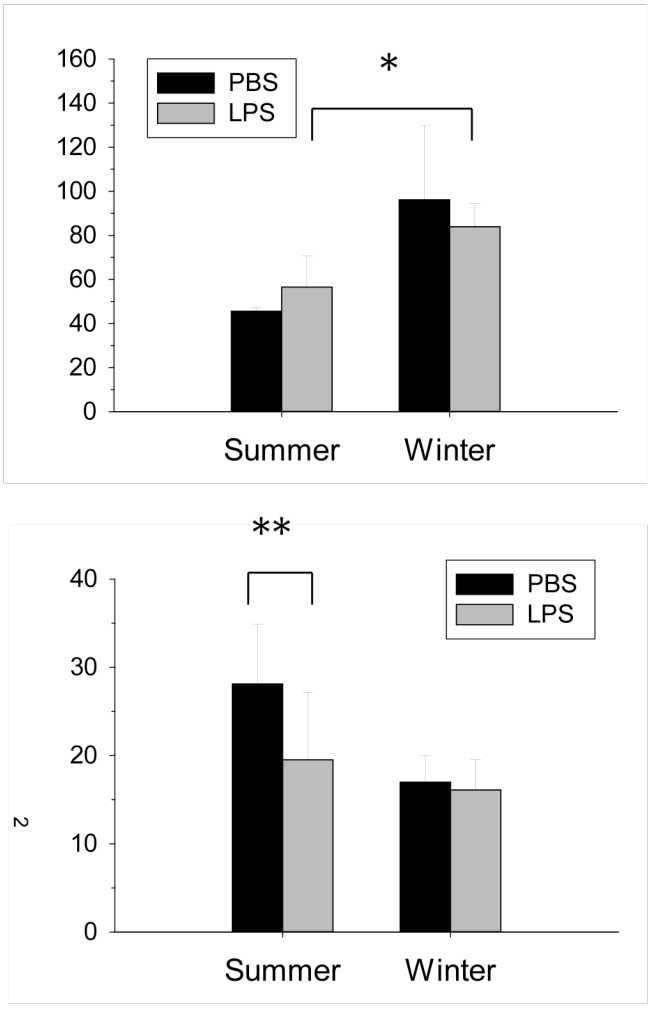

Figure 7. Mean breathing frequency and oxygen extraction efficiency ($E0_2$) under thermoneutral conditions in summer and winter black-capped chickadees injected with either PBS or LPS.

* Indicates significant difference between seasons ($P<0.05$)
** Indicates significant differences between treatment ($P<0.05$)

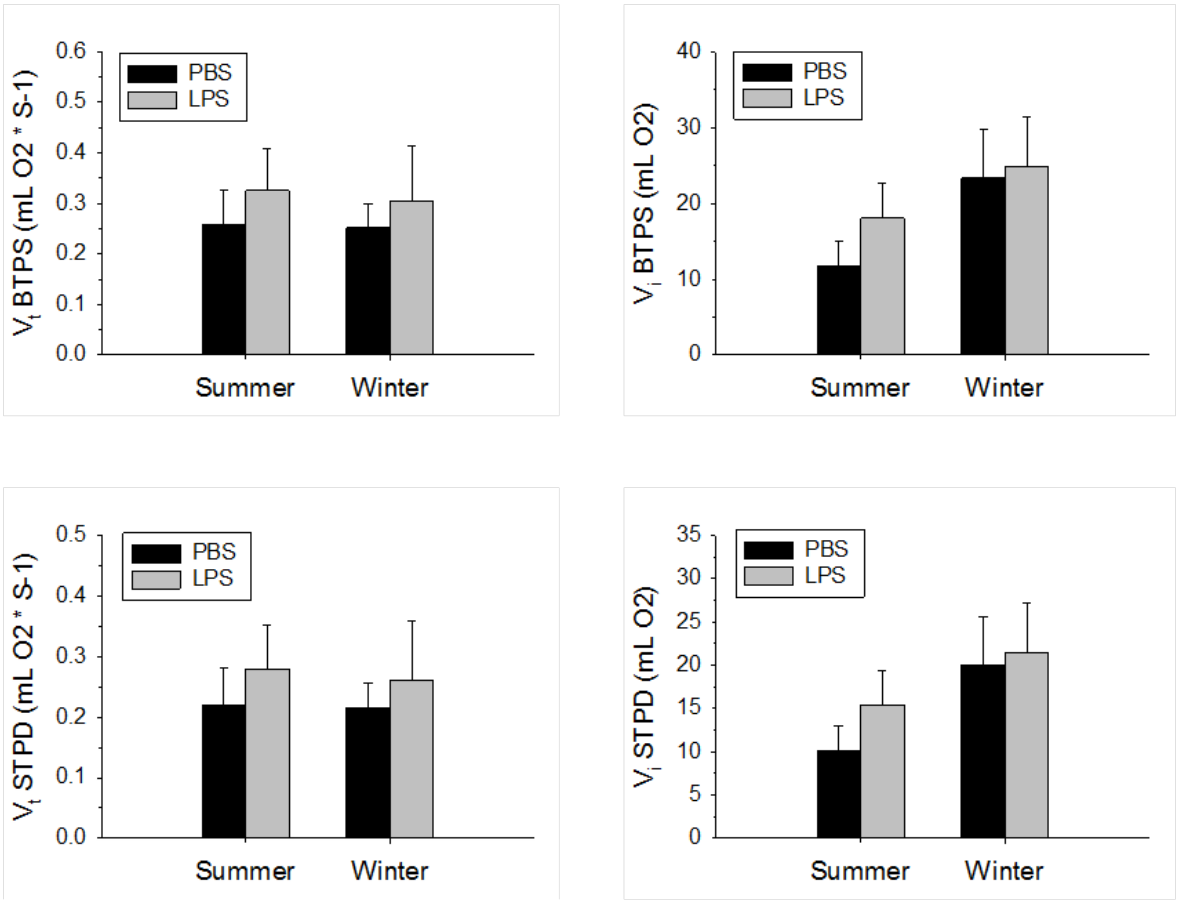

Figure 8. Mean tidal volume (V_t) BTPS, mean tidal volume (V_t) STPD, mean minute volume (V_i) BTPS, and mean minute volume (V_i) STPD under thermoneutral conditions in summer and winter black-capped chickadees injected with either PBS or LPS.

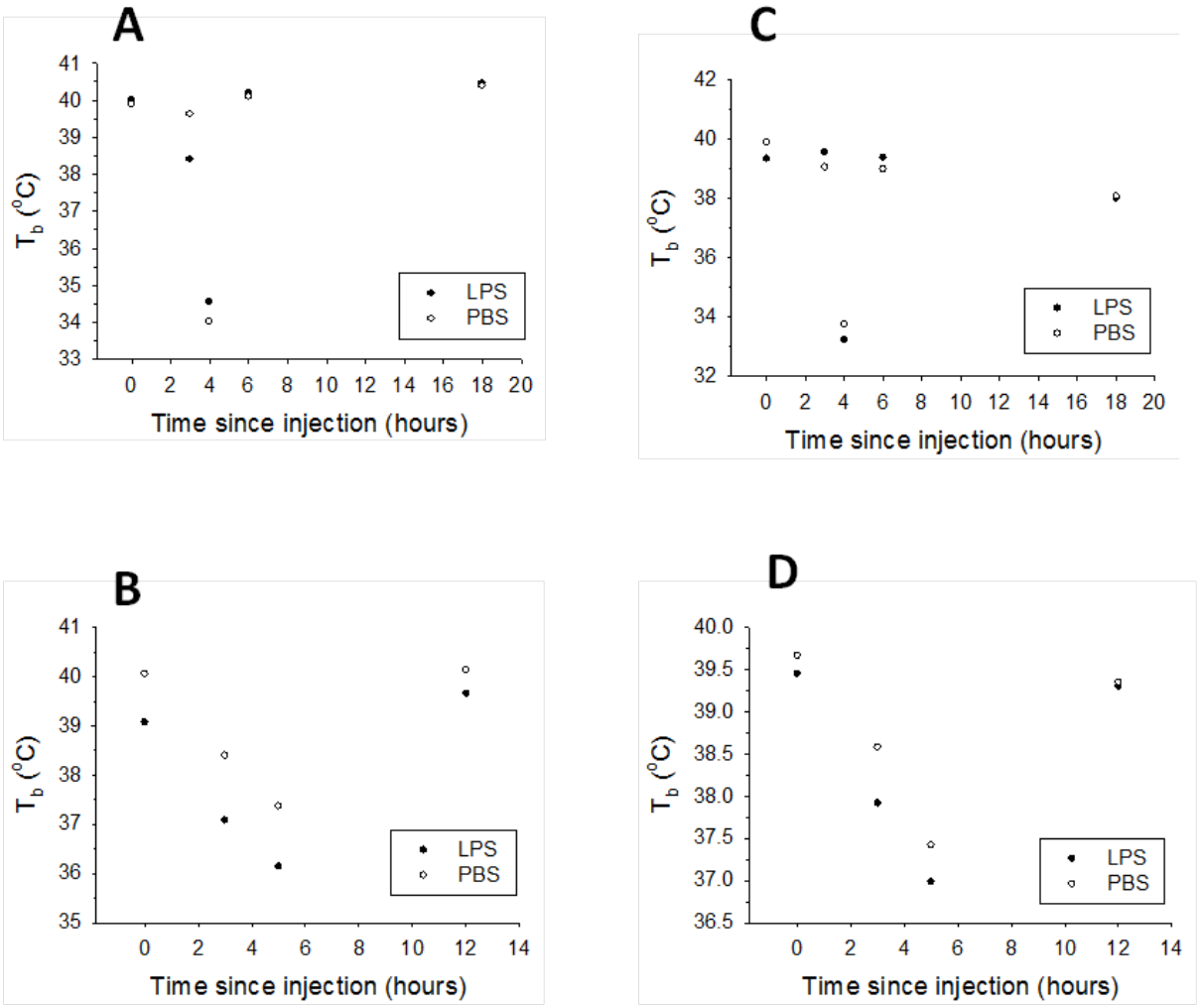

Figure 9. **A.** Mean body temperatures (T_b) during helox cold stress in summer black-capped chickadees. **B**. Mean body temperatures (T_b) under thermoneutral conditions in summer black-capped chickadees. **C.** Mean body temperatures (T_b) during helox cold stress in winter black-capped chickadees. **D.** Mean body temperatures (T_b) under thermoneutral conditions in winter black-capped chickadees.

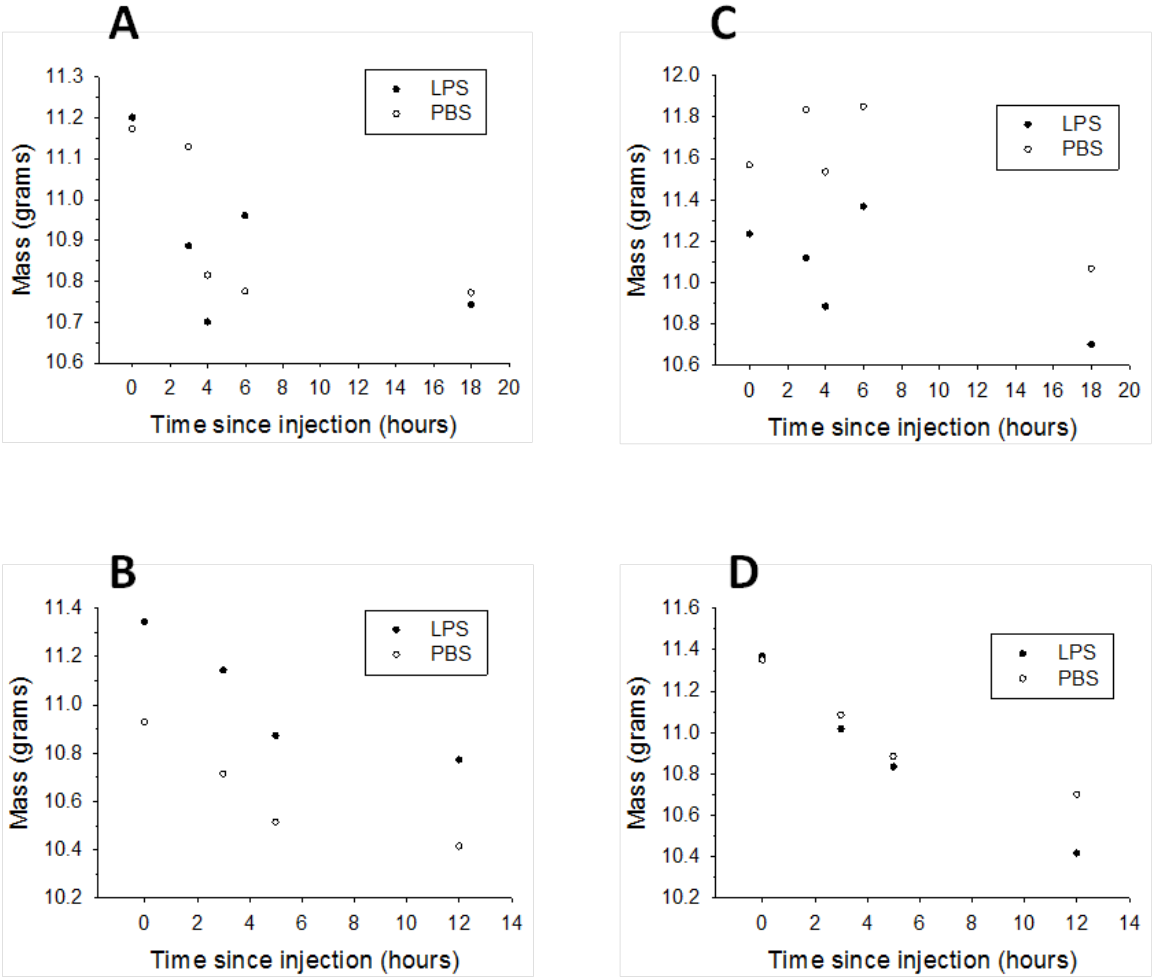

Figure 10. **A.** Mean masses during helox cold stress in summer black-capped chickadees. **B**. Mean masses under thermoneutral conditions in summer black-capped chickadees. **C.** Mean masses during helox cold stress in winter black-capped chickadees. **D.** Mean masses under thermoneutral conditions in winter black-capped chickadees.

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