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IMPACTS OF HUNTING AND LIFE-HISTORY STAGE ON THE STRESS PHYSIOLOGY AND BODY CONDITION OF FALL AND WINTERING MALLARDS (*ANAS PLATYRHYNCHOS*)

by

Jerad Richard Henson

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ABSTRACT

Henson, Jerad Richard. Ph.D. The University of Memphis. December 2017. Impacts of Hunting and Life-History Stage on the Stress Physiology and Body Condition of Fall and Wintering Mallards (*Anas platyrhynchos*). Co- Major Professors: Stephan J. Schoech, Ph.D. and David A. Freeman, Ph.D.

Waterfowl face a multitude of stressors across the fall and winter. These stressors include energetic demands associated with annual cycle stage, weather, habitat availability, and waterfowl hunting seasons. Stressful stimuli elicit a physiologic stress response culminating with the release of corticosterone (CORT). CORT aids in survival and recovery over the short-term, but if elevated over a long period it can lead to decrements in health. To avoid the potential harmful effects of prolonged elevations in CORT, some birds seasonally dampen their response to a predictable stressor. The aim of this study was to examine the changes in stress physiology and body condition of Mallards (*Anas platyrhynchos*) across the fall and wintering period. Mallards were sampled via netting or lethal take pre-migration in North Dakota and across the fall and winter in eastern Arkansas. Netted Mallards underwent a standard capture and handling protocol with blood samples taken over an hour, whereas shot Mallards had a blood sample taken immediately. Blood samples were used for plasma CORT and triglyceride (TRIG) analysis. Body mass, body condition index (BCI, mass corrected for size), and TRIG were regressed against subcutaneous fat thickness to determine which was the best indicator of fat deposits and therefore condition. While all three measures were significantly correlated with fat, BCI explained the most variation in fat deposits and therefore was used as the primary factor for assessing condition. Baseline CORT levels did not change across seasons, but body condition was reduced in Mallards across the fall into winter, and was lowest during the hunting season. Mallards had a reduced CORT response during fall migration and an increased response during the late winter, when Mallards complete pair formation. These results are similar to other species in which there were no changes in baseline CORT across seasons: a dampened CORT response during the energetically expensive periods of migration and molt, and increased responsiveness associated with breeding behaviors.

PREFACE

The research goal of my lab is to understand how birds cope with a changing world. Mallards, *Anas platyrhynchos*, are an excellent model organism for this research as they face many predictable and unpredictable stressors across their annual cycle. Mallards are the most abundant waterfowl species in North America. During the fall, Mallards leave their breeding grounds in Canada and the north central United States and head south to habitats with more readily available resources and milder temperatures. Although Mallards are present in all of the North American flyways, the Mid-continent breeding population, consisting of those birds that use the Central and Mississippi flyways, comprise the majority of the population. The wintering destination for most Mid-continent Mallards has historically been the Mississippi Alluvial Valley (MAV). While in the MAV, Mallards may experience many challenges, both predictable and unpredictable. The former are characterized by an increased energetic demand associated with various stages of the annual cycle, such as southward migration, pre-basic molt, pre-alternate molt, and preparation for spring and fall migration. Examples of unpredictable challenges include severe weather events, limited or degraded habitat availability, and numerous predators, including waterfowl hunters. How an individual responds to these challenges can have lasting carry-over effects on that individual's overall physical condition, reproductive capability, and social status. Thus, understanding the endocrine mechanisms mediating the response of an individual to such challenges is important for making informed decisions to maintain the health and facilitate the management of this species. I am the primary author on all three of these co-authored manuscripts. Chapter 1 (Introduction) has been formatted according to the style of *Condor*. Chapter 2 (Plasma

Triglycerides as Predictors of Body Fat in Mallards (*Anas platyrhynchos*)) has been formatted for submission to the *Journal of Field Ornithology*. Chapter 3 (Effects of Hunting on Baseline Stress Physiology and Body Condition in Waterfowl) has been formatted for submission to the *Condor*. Chapter 4 (Mallards (*Anas platyrhynchos*) Regulate Stress Responsiveness According to Energetic Demands During the Fall and Winter) has been formatted for submission to the *Journal of General and Comparative Endocrinology*. Chapter 5 (Summary of Conclusions) has been formatted according to the style of *Condor.*

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CHAPTER 1: INTRODUCTION

Waterfowl experience numerous environmental challenges throughout their annual cycle. These challenges include a suite of factors that increase energetic demand on an individual and which, in turn, induce physiological changes to allow the individual to meet said challenges. Examples include molt, migration, reproduction, food deprivation, a predation attempt, and inclement weather. The ability to survive and cope with such challenges has clear fitness implications (i.e., survival and successful reproduction).

Birds respond to energetic challenges by eliciting a physiological stress response which results in the release of catecholamines and glucocorticoids. Activation of the sympathetic nervous system is among the earliest responses to stressful stimuli, resulting in a rapid, within seconds release of catecholamines (epinephrine and norepinephrine, the *fight-or-flight* response). Catecholamines act both through direct release at specific target tissues, as well as systemic release into the circulatory system. Shortly thereafter, activation of the hypothalamic-pituitary-adrenal (HPA) axis culminates in a prolonged increase in circulating plasma glucocorticoids (Siegel, 1980; Wingfield et al., 1998; Sapolsky et al., 2000; Romero et al., 2009). Corticosterone (CORT), the primary avian glucocorticoid, is secreted from the adrenal cortex within minutes of a stressful encounter (Romero and Romero, 2002; Romero and Reed, 2005; Small et al., 2017). Elevated CORT levels associated with a stress response initiate multiple changes in behavior and physiology, partly via permissive actions to facilitate catecholamine functions (Sapolsky et al., 2000). Wingfield et al. (1998) outlined the effects of a CORT response in birds in which nonessential activities, such as reproductive, territorial, and social behaviors, are inhibited in favor of those behaviors that facilitate surviving the stressor. The immediate effects of elevated CORT, in support of catecholamine functions, are largely metabolic, and include increases in heart rate, blood pressure, and blood glucose levels (Sapolsky et al., 2000). Longer acting effects include suppression of reproductive behaviors, down regulation of the immune system, increased gluconeogenesis (through protein and fat catabolism), increased foraging behavior, promotion of irruptive behavior during the day, reduced metabolic rate at night (with the exception of times of nocturnal migration), and promotion of recovery upon cessation of the stressor (for review see Wingfield et al., 1998). All of these responses aid in survival and the reestablishment of homeostasis.

The duration of exposure to a stressor determines whether the activation of the HPA axis will be beneficial or harmful. In general, a stress response occurs over a relatively short period of time (minutes to hours). Once the challenge or stressor is removed, CORT levels return to baseline in roughly one hour (Romero and Romero, 2002). This type of stress response, which might be mounted in response to an attack by a predator or a territorial dispute, is considered an *acute* response and is considered “adaptive” because it increases the likelihood of survival, as well as promoting improvements in social standing, memory, and health (Siegel, 1980; Wingfield et al., 1998; Creel, 2001; Boonstra, 2004; Schoech et al., 2011; Jones et al., 2016). Conversely, long-term CORT elevation, due to exposure to a prolonged or frequently occurring stressor, reflects *chronic* stress which can lead to decrements in health, cognitive performance, and social standing, as well as delayed onset (or the abandonment) of reproduction (Siegel, 1980; Wingfield et al., 1998; Boonstra, 2004; Schoech et al. 2009, 2011).

During prolonged periods of stress, the body condition of birds deteriorates, an effect due in part to the up-regulation of the HPA axis (but see Cyr and Romero 2007, 2009). Whyte et al. (1984) found decreased lipid stores and increased blood glucose levels in Mallards (*Anas platyrhynchos*) during adverse weather conditions. Similar alterations in fat stores and blood glucose levels co-occur with elevated plasma CORT (Kitaysky et al., 1999; Perfito et al., 2002) and, given the known gluconeogenic and lipolytic actions of glucocorticoids (Remage-Healey and Romero, 2001), the decrement in condition is clearly due in part to the effects of CORT. It is possible, however, that alterations in behavior in response to stress, such as increased vigilance, could result in decreased time spent foraging and this could also negatively affect body condition (Breuner et al., 2008). The maintenance of condition is particularly important for migratory waterfowl from a conservation standpoint, because birds in better condition tend to have higher fitness and birds in poor body condition have an increased susceptibility to predation (Krapu, 1981; Bêty et al., 2003; Devries et al., 2008; Ankney et al., 2012; Juillet et al., 2012). In studies of recovery rates, Mallards that had been in poor condition when banded were more likely to be killed by hunters during the following hunting season (Hepp et al., 1986; Reinecke and Shaiffer, 1988). Also, the physical condition of female Mallards upon arrival at the breeding grounds is correlated with their ability to successfully reproduce, as individuals in better condition tend to progress through annual life history stages faster (Heitmeyer, 1988b, 2006). Thus, females in good condition in the fall and winter finish the pre-basic molt earlier and pair earlier than females in poor condition. The completion of the pre-basic molt in females in late winter is necessary for the initiation of spring migration (Dugger, 1997) and those that migrate earlier, nest earlier and have increased re-nesting opportunities (Devries et al., 2008).

Predation attempts, or even the perception of a predator, can cause elevations in plasma CORT levels (Silverin, 1998; Scheuerlein et al., 2001; Cockrem and Silverin, 2002; Jones et al., 2016) . Predation pressure on waterfowl is largely due to human hunting with over one million hunters entering the marshes, swamps, and prairies of the United States every fall and winter with the intent of harvesting waterfowl (Carver, 2008). Arkansas accounts for the largest annual Mallard harvest in the nation and because of this, it is a prime location for the study of these effects on Mallard physiology (Raftovich et al., 2011). It is important to understand not only how hunting affects Mallards, but also whether the duration and patterns of hunting pressure (season structure) affect Mallards. Hunters generate millions in revenue and conservation dollars annually, and understanding the impacts of hunting pressure on waterfowl is vital for the conservation of this resource.

Birds can mitigate potential effects of chronic stress by altering how they respond to a stressor (Romero et al., 1998). Many birds respond to predictable energetically demanding times by minimizing their CORT responsiveness (Romero et al., 1997, 1998; Romero, 2006; Wilson et al., 2004, 2017; Wingfield et al., 1995). Several species of birds dampen their stress response during times when elevated CORT may not be compatible with the increased demands of certain annual cycle stages (e.g., molt, reproduction, and migration; Romero et al., 1997, 1998; Romero, 2006; Wilson et al., 2004, 2017; Wingfield et al., 1995). Additionally, repeated or chronic exposure to stressful stimuli can lead to an overall decrease in CORT levels due to either exhaustion or perhaps as a protective mechanism (see Cyr and Romero, 2007, 2009).

The ability to measure body composition is extremely important for the assessment of body condition in birds as it facilitates an understanding of the impacts of fluctuations in both energy availability and energetic demands. Particularly important are lipid resources, given that they provide vital stores used to fuel birds during flight and while at rest (i.e., overnight). Lipid stores and reserves are the primary fuel during 1) long distance flight and other periods when feeding is impossible, such as during severe winter storms and 2) reproduction, especially in ‘capital’ breeders, as are many waterfowl species (Krapu, 1981; Ankney et al., 1991; Jenni and Jenni-Eiermann, 1998; Stephens et al., 2009). Mallards possess both of these characteristics. Hence, the ability to accurately determine fat deposits, which reflect condition, can be an invaluable tool for researchers and managers.

In the first study (Chapter 2) of this dissertation, I determined the best nonlethal measures that predict body fat, the primary indicator of condition in birds. A blood sample and morphometrics were taken from Mallards collected in Arkansas. Blood plasma was assayed for Triglycerides (TRIG) and morphometrics were used to create a body condition index (BCI). Thickness of fat on the breast was then measured and used to verify which measure of interest (TRIG, BCI or body mass) was the best indicator of fat depot during the fall and winter. In the second study (Chapter 3), the nature of links among body condition, CORT levels, and hunting were explored. Mallards were sampled pre-migration in North Dakota and across the fall and winter in eastern Arkansas. A blood sample was taken for baseline CORT analysis and a morphometric-derived body condition index was created for each bird to determine whether: 1) these measures varied across the nonbreeding season; 2) CORT and condition were intercorrelated; and 3) the presence or absence of hunting, or the duration of exposure to hunting (i.e., season length and temporal structure), impacted CORT and condition. In the third and final study (Chapter 4) I explored CORT responsiveness to a standardized stressor across life cycle stages and whether any changes were linked to body condition. Mallards were sampled during three periods at two locations: 1) fall, during pre-migration in North Dakota; 2) fall and early winter, during migration in Arkansas; and 3) late winter, during the early spring migration in Arkansas. Mallard CORT response and body condition were compared across sample periods to examined whether Mallards alter CORT responsiveness during the fall and winter and if so, whether this was influenced by body condition.

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CHAPTER 2: PLASMA TRIGLYCERIDES AS PREDICTORS OF BODY FAT IN MALLARDS (*ANAS PLATYRHYNCHOS*)

The ability to measure body composition is important for the assessment of body condition in birds as it facilitates an understanding of the impacts of fluctuations in both energy availability and energetic demands. Particularly important are lipid resources, given that they provide vital stores used to fuel birds during flight and while at rest (i.e., overnight). Lipid stores and reserves (see below for further discussion of the roles of *stores* versus *reserves*) are the primary fuel during 1) long distance flight and other periods when feeding is impossible, such as during severe winter storms and 2) reproduction – especially in ‘capital’ breeders, including many waterfowl species (Ankney et al., 1991; Jenni and Jenni-Eiermann, 1998; Krapu, 1981; Stephens et al., 2009). Hence, the ability to accurately determine fat deposits in living birds can be an invaluable tool for researchers and managers.

Fats are primarily stored subcutaneously as adipose tissue in discrete depots, but are also found within muscle and the body cavity (Blem, 1976). Fatty acids, synthesized from constituent acetyl-CoA and NADPH, combine with glycerol to form triglycerides that are then stored in adipose tissue. Adipose tissue provides eight to ten times as much energy per unit wet mass than other fuel types, and fatty acids provide twice the energy of carbohydrates and proteins per unit dry mass (McWilliams et al., 2004). Fat catabolism also provides metabolic water, 1.07 g water per 1 g of fat, which is vital during prolonged flight (Gill, 2007). Fat deposited in preparation for a predictable shortage in lipid-provided energy is termed “stores,” whereas endogenous fat used in times of stress are “reserves” (per Van der Meer and Piersma, 1994). Birds build fat stores both by hyperphagia and selecting diets higher in lipids, most commonly utilizing both of these strategies (McWilliams et al., 2004).

Numerous techniques have been used to measure or estimate lipid stores and reserves in birds. Methods include carcass analysis, morphology, fat scores, total body electrical conductivity, heavy water, and quantitative magnetic resonance (Brown, 1996; Guglielmo et al., 2011; Labocha and Hayes, 2012; Schoech, 1996; Speakman et al., 2001). Carcass analysis is by far the most common and accurate way to measure body composition; however, because it requires sacrificing the bird, it is not useful for longitudinal studies. For carcass analysis, the bird is dried, homogenized, and lipids are then extracted from a sample with an organic solvent (e.g., petroleum ether or chloroform). Accurate mass measurements of the animal before and after drying (‘wet’ vs. dry weight), and again of the fat-free animal (or a subsample thereof) allow the determinations of relative percentages of lean vs. fat composition. In contrast, there are several non-lethal methods for estimating body condition and fat deposits. Many researchers use morphometrics, including solely body mass or a body condition index (BCI; mass corrected for size) to estimate fat deposits in birds (Brown, 1996; Labocha and Hayes, 2012; Schoech and Bowman, 2003). One of the oldest methods for estimating fat depots, and one that is used in species with relatively transparent (translucent) skin, such as passerines and hawks, is fat scoring, in which adipose depot size in discrete subcutaneous regions of the interclavicular region and/or the abdomen is measured or estimated (Brown, 1996; Labocha and Hayes, 2012). Other methods used to estimate body fat include total body electrical conductivity (TOBEC), heavy water, and quantitative magnetic resonance (QMR). TOBEC measures percent lean body mass via conductance and fats are then computed by subtracting the estimated lean mass from the live total body mass (see Walsberg, 1988). Heavy water (deuterium) dilution provides an accurate measure of total body water, but measures fat indirectly and is time consuming (Speakman et al., 2001). QMR can accurately measure lean mass, fats, and total water and is one of the most accurate ways of assessing body condition without killing the birds (Guglielmo et al., 2011). Unfortunately, these latter three methods are quite costly and are difficult to use in the field, and all must be calibrated for the species of interest by comparing 'system derived' and carcass analysis values across a range of individuals (Labocha and Hayes, 2012; Guglielmo et al., 2011; Schoech, 1996).

Plasma metabolites have become a popular way to estimate body mass change of birds during migration, as well evaluate the habitat quality of stopover and over-winter sites (Anteau and Afton, 2008; Jenni-Eiermann and Jenni, 1994; Williams et al., 1999). As examples of the former, plasma triglycerides (TRIG) are elevated during times of fat deposition and β-hydroxybutyrate is elevated during fasting (Jenni-Eiermann and Jenni, 1994). The ratio of TRIG to β-hydroxybutyrate has been used in Lesser Scaup (*Aythya affinis*), Garden Warblers (*Sylvia borin*), and Western Sandpipers (*Calidris mauri*) to determine whether a bird is in a state of anabolism or catabolism of body fats at stopover sites (Anteau and Afton, 2008; Jenni-Eiermann and Jenni, 1994; Williams et al., 1999; respectively). These measures are useful and convenient because they can be determined using straightforward laboratory analysis of a blood sample.

Mallards (*Anus platyrhynchos*) are migratory capital breeders that depend heavily on fat stores and reserves to progress efficiently through the annual cycle. However, given their thick skin and heavy layer of down, estimating fat scores of waterfowl per the methods used in song birds (see above) is not possible. Thus, the only way to accurately determine the body lipid content of waterfowl is to do a post mortem body composition analysis (Drobney, 1982), or using expensive (QMR) equipment (see above discussion and citations).

It is well known that TRIG increase during anabolism as birds build fat stores; however, the precise relationship between TRIG and fat depots is unknown. The primary aim of this study was to determine whether TRIG could be used to estimate the lipid stores and reserves of Mallards. Secondarily, we wished to determine the utility of TRIG as compared to two other commonly used non-lethal measures of estimating body fat - body mass and a body condition index. Lastly, we consider whether use of TRIG in concert with either body mass or a BCI provided a better estimator of our fat measure. We predicted that TRIG would be positively correlated with fat deposits. However, we were uncertain whether the TRIG:fat relationship would be strong enough to make it a useful addition or an accurate tool for estimating lipids. That said, if shown to be useful, assay of TRIG could be a cost effective, quick, and non-lethal method for assessing fat depots in Mallards and other species.

METHODS

Study Population and Data Collection

Mallards were collected during the migrating and wintering phases of their annual cycle from November 19, 2014 through February 20, 2015. All collection sites were flooded, bottomland hardwood forest, as well as rice and soybean fields, and located throughout Jackson, Monroe, Crittenden, and Desha counties in eastern Arkansas.

Collection was done by lethal take with a shotgun (n = 64). Because of blood sample collection problems associated with clotting (JRH pers. obs.), blood samples were collected within 5 min (n = 61). A dead Mallard was laid on its back and the axillary artery and veins were severed allowing cardiac blood to drain into a pool on the wing, from which a sample was collected in heparinized capillary tubes. In all instances, samples were kept on ice until they could be centrifuged (2 - 5 hr) and the plasma drawn off. Plasma was stored at -20˚ C until assay.

Following sample collection, mass and wing cord length were recorded and a body condition index (BCI) was generated using the method of Peig and Green (2009, 2010). Sex was determined based on plumage characteristics. The thickness of an individual's subcutaneous, breast fat depot was measured by plucking a portion of the breast, and making an incision one inch lateral from the keel and one inch below the furcula (i.e., from the rostrum of the carina, in close proximity to the flattened fusion-point of the two clavicles that make-up the furcula). This was done in lieu of a body composition analysis for two reasons. First, resources were not available to process and carry out carcass analysis (see Drobney, 1982, Krapu, 1981). Second, most samples were taken from hunter harvested birds; therefore, after a blood sample and morphometrics were collected, the birds were returned to the hunter. All research was done under USGS banding permit, USFWS scientific collection permit, and AGFC scientific collection permits.

Sample Analyses

TRIG was quantified using 10 µl of plasma and a Triglyceride Colorimetric Assay Kit from Cayman Chemical, Ann Arbor, MI (Cat No. 10010303). Each sample was run in duplicate and a standard curve from 0 mg/dl to 200 mg/dl was generated using a known concentration of triglycerides. Most plasma samples were undiluted; however, a 1:2 dilution was used in cases in which a value was off the curve. Intra-assay variation was below 12% for all runs and averaged 5.6%, inter-assay variation was 5%.

Statistical analyses

To determine which of the measures of interest (BCI, body mass, and TRIG) was the best predictor of fat depot thickness (our proxy for total body lipids), a linear regression was run for each measure. The best model from our measures of interest was determined by the model with the highest R^2. A multiple regression adding TRIG to body mass or BCI was used to determine if TRIG provided additional power over just the morphometric measures. BCI was not normally distributed; therefore, BCI was $\log_{10}$ transformed for statistical analysis. All analyses were carried out in R.

RESULTS

The separate regressions revealed that each measure (body mass, BCI, and TRIG) was significantly correlated with fat deposits on the breast. BCI was the best single predictor of fat thickness on the breast, followed by body mass, then TRIG (Table 1). TRIG and BCI together was the best model and explained the most variance in the thickness of the breast fat depot (Table 1; $F_{2,60} = 17.33$, $R^2 = 0.37$, $p = 7.8e\text{-}05$). Mallards with larger fat deposits also had higher TRIG levels, but the addition of TRIG to the model minimally increased the model's ability to explain the variance in the data. All three predictors are independently graphed below (Fig.1 - body mass, Fig. 2 - BCI, and Fig. 3 - TRIG).

Table 1. Summary of regression results of body mass, BCI, and TRIG against fat thickness.

	F	**df**	***p***	***R^2***
Body Mass	12.89	1,64	0.0006*	0.17
BCI	25.38	1,64	4.1e-06*	0.28
TRIG	4.368	1,61	0.041*	0.067
Body Mass + TRIG	11.13	2,60	7.8e-05*	0.27
BCI + TRIG	17.33	2,60	1.146e-06*	0.37

* Indicates statistical significance.

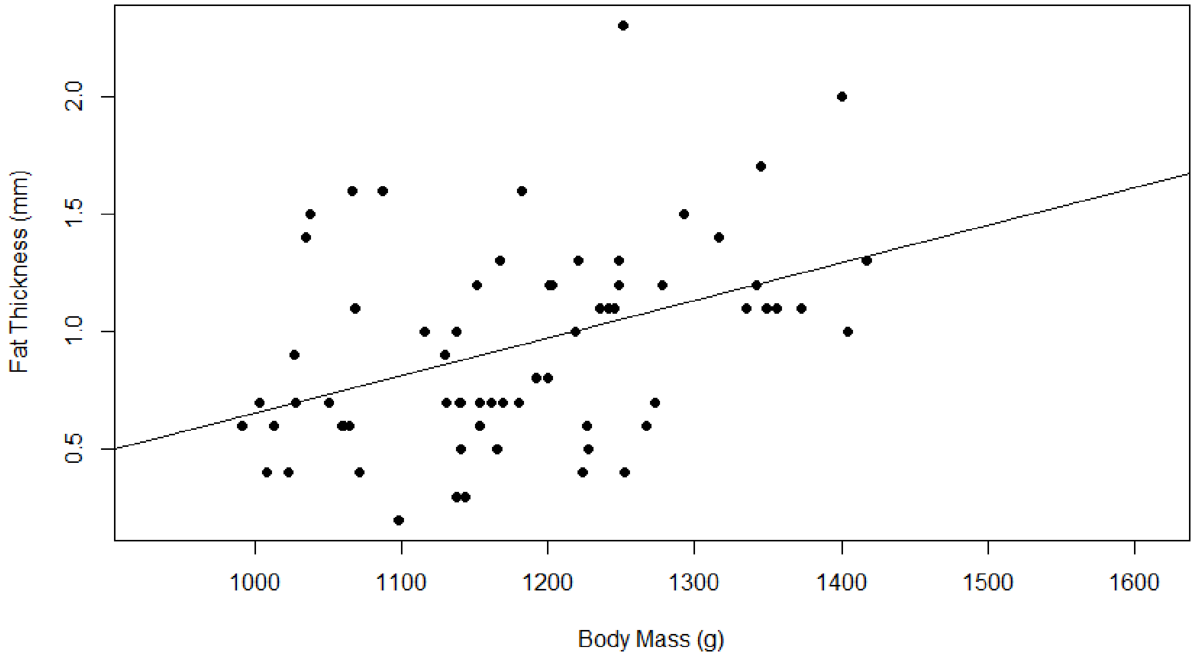

Figure 1. Subcutaneous fat thickness on the breast regressed against body mass with trend line. See Table 1 for statistical values.

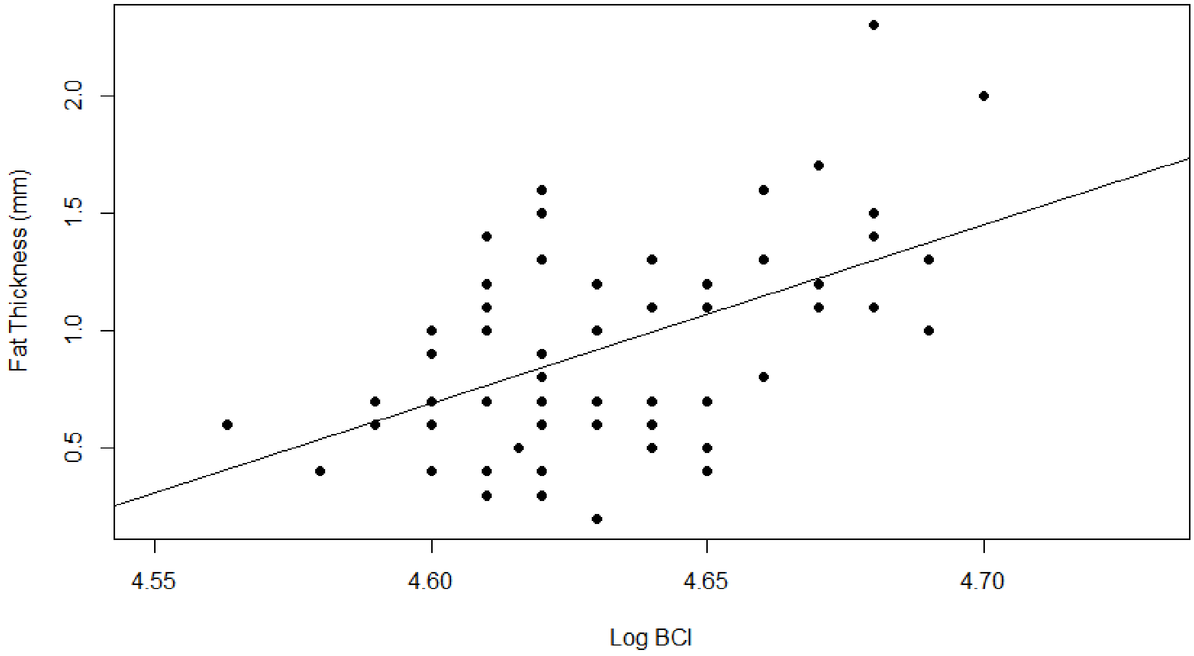

Figure 2. Subcutaneous fat thickness on the breast regressed against body condition index with trend line. See Table 1 for statistical values.

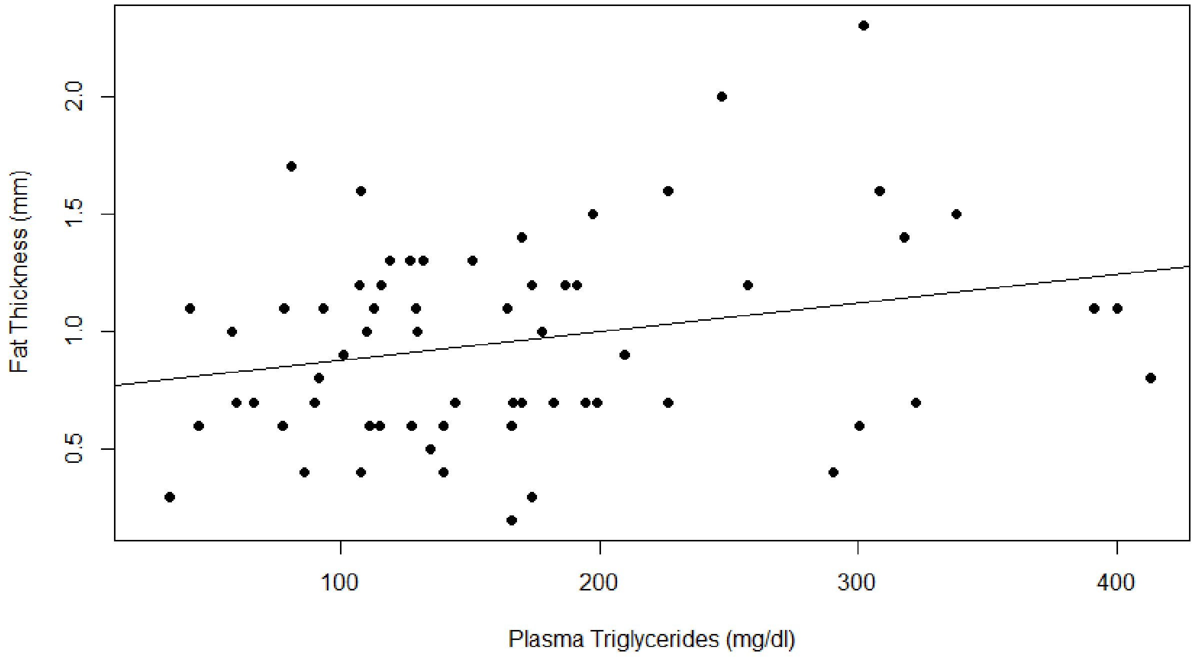

Figure 3. Subcutaneous fat thickness on the breast of Mallards plotted against plasma triglycerides with trend line. See Table 1 for statistical values.

DISCUSSION

Our results show that body mass, BCI, and TRIG all were positively correlated with breast fat thickness in Mallards (Table 1; Fig. 1, 2, and 3). This agrees with previous work on Mallards indicating that body mass and BCI are correlated with body fat (Boos et al., 2000, 2002; Whyte and Bolen, 1984; Whyte et al., 1986). Interestingly however, both body mass and BCI were relatively weakly correlated with our measure of fat deposits, although both have been used as strong indicators ($R^2 \geq 0.49$) of total body fat in other studies of Mallards during fall and winter see Boos et al., 2000, 2002; Labocha and Hayes, 2012; Whyte and Bolen, 1984; Whyte et al., 1986).

While TRIG were positively correlated with breast fat depot thickness, they offer minimal predictive value for assessing body fat in Mallards ($R^2 = 0.067$). Labocha and

Hayes (2012) argue that a multiple regression model with multiple measures regressed against total body fat is the best predictor. Our data offer some support for this hypothesis, as a multiple regression model including BCI and TRIG best explained variance in our measure of body fat ($R^2 = 0.37$). However, it is questionable whether the added expense and effort involved in quantifying TRIG is justified, given the minimal additional variance explained by its inclusion in the model. Indeed, a well-considered BCI (see Schoech, 2009; review in Labocha and Hayes, 2012) alone might be the best option.

TRIG may be more useful as an indicator of ongoing lipid mass change (i.e., anabolism vs. catabolism) rather than an estimate of nutrient stores or reserves (Anteau and Afton, 2008; Jenni-Eiermann and Jenni, 1994). Anteau and Afton (2008) found that TRIG and β-hydroxybutyrate explained 75% of daily mass change in Lesser Scaup. Ideally, a better understanding of the daily and seasonal flux in the condition of Mallards and whether this varies across habitats would not be based upon a single static measure. Rather, an ideal approach might include a suite of both physiological variables and morphometrics, revealing the direction of changes and any interactions among measures across time. Ideally, future studies on Mallards would include β-hydroxybutyrate as well as TRIG, as these combined measures would provide a better understanding of their relative roles, inter-relationship, and flux across catabolic and anabolic stages. This would provide an in depth look at the nature and inter-relationships of multiple indicators of body condition across the annual cycle and among habitats (Anteau and Afton, 2008; Jenni-Eiermann and Jenni, 1994).

The relatively weak relationships between both BCI and body mass and fat depot thickness in the current study were considerably lower than those of previous studies on Mallards (Boos et al., 2000, 2002; Labocha and Hayes, 2012; Whyte and Bolen, 1984; Whyte et al., 1986). One factor that could contribute to this disagreement between our and other studies is that the subcutaneous fat depot we used may not accurately reflect total body fat stores. Although Mallards and other birds primarily deposit fat stores subcutaneously (Blem, 1976; Boos et al., 2002), body cavity abdominal fat can make-up a significant proportion of the total body fat (Bailey, 1979; King, 1976; Wishart, 1979). In our study, it seems as if breast fat thickness and total body fat are not characterized by a one-to-one relationship. Clearly, further research is needed to elucidate the proportional relationships amongst the various sites where fats are stored.

In conclusion, our findings suggest that BCI and TRIG can be used to roughly estimate fat deposits in Mallards. That said, BCI alone offered the simplest method for estimating fat deposits and the little improvement with TRIG incorporated in the model likely doesn’t justify the additional expense and effort. While a multiple regression analysis that included both BCI and TRIG was the best predictor of the thickness of the breast fat, the inclusion of the metabolite, β-hydroxybutyrate in the analysis would give a better picture of the catabolic/anabolic state of the animal. Additionally, an even better measure of the status of an individual could be gained by measuring repeated samples, although obtaining such in free-living Mallards would be difficult.

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CHAPTER 3: EFFECTS OF HUNTING ON BASELINE STRESS PHYSIOLOGY AND BODY CONDITION IN WATERFOWL

INTRODUCTION

Waterfowl experience numerous physiological challenges throughout their annual cycle. These challenges include a suite of factors that increase energetic demand on an individual. For example, molt, migration, reproduction, food deprivation, a predation attempt, and inclement weather. The ability to survive and cope with such challenges has clear fitness implications (i.e., survival and successful reproduction).

The most abundant waterfowl species in North America is the Mallard (*Anas platyrhynchos*). During the fall, Mallards leave their breeding grounds in Canada and the north central United States and head south to habitats with more readily available resources and milder temperatures. Although Mallards are present in all of the North American flyways, the Mid-continent breeding population, consisting of those birds that use the Central and Mississippi flyways, comprise the majority of the population (US Fish and Wildlife Service, 2016). The wintering destination for most Mid-continent Mallards has historically been the Mississippi Alluvial Valley (MAV) in the Mississippi flyway, comprised of portions of Missouri, Arkansas, Mississippi, and Louisiana along the lower Mississippi River (Bellrose, 1976; Reinecke et al., 1989). While in the MAV, Mallards may experience many challenges, both predictable and unpredictable. The former are characterized by an increased energetic demand associated with various stages of the annual cycle, such as southward migration, pre-basic molt, pre-alternate molt, and preparation for spring and fall migration (Heitmeyer, 1988a; 1988b; 2006; Richardson & Kaminski, 1992; Dugger, 1997). Examples of unpredictable challenges include severe weather events, limited or degraded habitat availability, and numerous predators, including waterfowl hunters. How an individual responds to these challenges, both predictable and unpredictable, can have lasting carry-over effects on that individual's overall physical condition, reproductive capability, and social status. Thus, understanding the endocrine mechanisms mediating the response of an individual to such challenges is important for making informed decisions to maintain the health and facilitate the management of this species.

Activation of the sympathetic nervous system is among the earliest responses to stressful stimuli, resulting in a rapid increase in catecholamine (epinephrine and norepinephrine; the *fight-or-flight* response) release (i.e., both direct release at specific target tissues, as well as systemic release into the circulatory system). This is followed shortly thereafter by activation of the hypothalamic-pituitary-adrenal (HPA) axis, which culminates in a prolonged increase in circulating plasma glucocorticoids (Siegel, 1980; Wingfield et al., 1998; Sapolsky et al., 2000; Romero et al., 2009). Corticosterone (CORT), the primary avian glucocorticoid, is secreted from the adrenal cortex within minutes of a stressful encounter (Romero & Romero, 2002; Romero & Reed, 2005; Small et al., 2017). Elevated CORT levels associated with a stress response initiate multiple changes in behavior and physiology, partly via permissive actions to facilitate catecholamine functions (Sapolsky et al., 2000). Wingfield et al. (1998) outlined the effects of a CORT response in birds in which nonessential activities, such as reproductive, territorial, and social behaviors, are inhibited in favor of those behaviors that facilitate surviving the stressor. The immediate effects of elevated CORT, in support of catecholamine functions, are largely metabolic, and include increases in heart rate, blood pressure, and blood glucose levels (Sapolsky et al., 2000). Longer acting effects include suppression of reproductive behaviors, down regulation of the immune system, increased gluconeogenesis (through protein and fat catabolism), increased foraging behavior, promotion of irruptive behavior during the day, reduced metabolic rate at night (with the exception of times of nocturnal migration), and promotion of recovery upon cessation of the stressor (for review see Wingfield et al., 1998). All of these responses aid in survival and the reestablishment of homeostasis.

The duration of exposure to a stressor determines whether the activation of the HPA axis will be beneficial or harmful. In general, a stress response occurs over a relatively short period of time (minutes to hours). Once the challenge or stressor is removed, CORT levels return to baseline in roughly one hour (Romero and Romero, 2002). This type of stress response, which might be mounted in response to an attack by a predator or a territorial dispute, is considered an *acute* response and is considered “adaptive” because it increases the likelihood of survival, as well as promoting improvements in social standing, memory, and health (Siegel, 1980; Wingfield et al., 1998; Creel, 2001; Boonstra, 2004; Schoech et al., 2011; Jones et al., 2016). Conversely, long-term CORT elevation, due to exposure to a prolonged or frequently occurring stressor, reflects *chronic* stress which can lead to decrements in health, cognitive performance, and social standing, as well as delayed onset (or the abandonment) of reproduction (Siegel, 1980; Wingfield et al., 1998; Boonstra, 2004; Schoech et al. 2009, 2011).

During prolonged periods of stress, the body condition of birds deteriorates, an effect due in part to the up-regulation of the HPA axis (but see Cyr and Romero 2007, 2009). Whyte et al. (1984) found decreased lipid stores and increased blood glucose levels in Mallards during adverse weather conditions. Similar alterations in fat stores and blood glucose levels co-occur with elevated plasma CORT (Kitaysky et al., 1999; Perfito et al., 2002) and, given the known gluconeogenic and lipolytic actions of glucocorticoids (Remage-Healey & Romero, 2001), the decrement in condition is clearly due in part to the effects of CORT. It is possible, however, that alterations in behavior in response to stress, such as increased vigilance, could result in decreased time spent foraging and this could also negatively affect body condition (Breuner et al., 2008). The maintenance of condition is particularly important for migratory waterfowl from a conservation standpoint, because birds in better condition tend to have higher fitness and birds in poor body condition have an increased susceptibility to predation (Krapu, 1981; Bêty et al., 2003; Devries et al., 2008; Ankney et al., 2012; Juillet et al., 2012). In studies of recovery rates, Mallards that had been in poor condition when banded had increased probabilities of being killed by hunters during the following hunting season (Hepp et al., 1986; Reinecke and Shaiffer, 1988). Also, the physical condition of female Mallards upon arrival at the breeding grounds is correlated with their ability to successfully reproduce, as individuals in better condition tend to progress through annual life history stages faster (Heitmeyer, 1988b, 2006). Thus, those females that are in better condition in the fall and winter finish the pre-basic molt earlier and pair earlier as compared to females in worse condition. The completion of the pre-basic molt in females in late winter is necessary for the initiation of spring migration (Dugger, 1997) and those that migrate earlier, nest earlier and have increased re-nesting opportunities (Devries et al., 2008).

Predation attempts, or even the perception of a predator, can cause elevations in plasma CORT levels (Silverin, 1998; Scheuerlein et al., 2001; Cockrem and Silverin, 2002; Jones et al., 2016) . Predation pressure on waterfowl is largely due to human hunting. Over one million hunters enter the marshes, swamps, and prairies of the United States every fall and winter with the intent of harvesting waterfowl (Carver, 2008). Arkansas accounts for the largest annual Mallard harvest in the nation and because of this, it is a prime location for the study of these effects on Mallard physiology (Raftovich et al., 2011). It is important to understand not only how hunting affects Mallards, but also whether the duration and patterns of hunting pressure (season structure) affect Mallards. Hunters generate millions in revenue and conservation dollars annually, and understanding the impacts of hunting pressure on waterfowl is vital for the conservation of this resource.

This study aims to determine if Mallard stress physiology and body condition vary across the nonbreeding season and the degree to which hunting or hunting duration contributes to any observed variations. We hypothesize that hunting pressure affects baseline plasma CORT levels and predict that hunted birds will have higher baseline CORT levels than non-hunted birds. We also hypothesize that the duration of hunting will alter baseline plasma CORT levels and body condition, and expect that as hunting duration increases, baseline CORT levels will increase and body condition will decrease.

METHODS

Study Populations

Migrating and wintering Mallards were sampled in flooded bottomland hardwood forest and flooded rice and soybean agricultural fields in Jackson, Monroe, Crittenden, Drew, and Desha counties in eastern Arkansas over the course of four years (2013-2017). Fall samples were collected from pre-migratory Mallards in the Souris River valley at J. Clark Salyer National Wildlife Refuge in North Dakota in September of 2016.

Data Collection

Mallards were sampled in two ways, via capture in nets and lethal take with a shotgun (n=136, 16 netted and 120 lethal take). Samples were collected during 4 periods: fall pre-migration (September 16th -21st), migration pre-hunting season (November 1st – Friday before Arkansas waterfowl hunting opener in mid-November), migration/wintering during hunting season (Arkansas hunting season, mid-November – end of January), and spring pre-migration (February – March). Sample periods allow us to observe any physiological changes in baseline CORT or body condition pre-, during, and post- hunting season in Arkansas. The fall pre-migration sample period provides a control group before Mallards can be hunted in any US state and allows a better understanding of how fall migration might affect Mallards. All fall pre-migration samples were obtained from birds that were netted, whereas all samples during the hunting season were lethal take by hunters. Netting Mallards during hunting season proved difficult due to federal regulations that prohibited the use of bait during hunting season. Pre- and post-hunting season samples were a mix of netted and lethal take birds and all lethal take samples at this time were sampled using the same hunting methods as birds sampled during the hunting season (attracted using decoys). Lethal take sampling allows an insight into true baseline plasma CORT levels. Because CORT takes approximately 2-3 min to enter the blood stream after the initiation of a stress response (Romero and Reed, 2005; Small et al., 2017), a sample collected before this will be independent of a CORT response due to capture (i.e., the immediate death of an individual eliminates any possibility of a CORT response). Collection of samples from shot Mallards presumably provides a true time '0' blood sample; whereas, a netted bird would not be sampled until 1-2.5 minutes post capture (see Small et al., 2017).

Mallards were caught using mist nets, rocket nets, or a net launcher (Coda Netgun; Coda Enterprises, Mesa, AZ) and were subjected to a standardized capture and handling stress protocol that simulates an acute stressor (see Wingfield et al., 1992). A blood sample was taken within 2.5 minutes for baseline samples. Blood samples were taken from the brachial vein (140 – 280 µl) using a 25 gauge needle and heparinized capillary tubes.

Because of sample collection problems associated with clotting (JRH pers. obs.), lethal take Mallards were sampled within 5 min of being shot. Only birds for which pellets caused instantaneous death from a single shot were sampled. Birds were sampled by laying them on their back and cutting the axillary artery and vein which would drain cardiac blood into a pool on the wing that could be collected with heparinized capillary tubes. In all instances, samples were kept on ice until they could be centrifuged (2-5 hrs) and the plasma drawn off. Plasma was stored at -20˚C until assay.

Following sample collection and before release, netted birds received a USGS leg band, were sexed based on plumage, and mass and wing cord length were also recorded.

A body condition index (BCI) was generated using the morphometric methods of Peig and Green (2009, 2010). Climate data was recorded from the nearest NOAA weather station. High and low temperatures were recorded for the day of sampling to control for effects on baseline CORT and BCI (Whyte and Bolen, 1984; Schoech et al., 2011). All research was done under USGS banding permit #23254, USFWS scientific collection permit #MB49809B-1, AGFC scientific collection permit #011320172, and ND scientific collection permit #GNF04121011.

Sample Analyses

We quantified baseline plasma CORT using 10-15 µl of plasma. Samples were assayed with a CORT ELISA from Cayman Chemical, Ann Arbor, MI (Cat No. 500655). This kit has been validated in our lab for use in waterfowl (Wilson et al., 2017). Each sample was run in duplicate and a standard curve from 5000 pg/ml to 8.2 pg/ml was generated using a known concentration of CORT. Each plate contained internal controls (high IC 600 pg/ml at approximately 20% binding; low IC 60 pg/ml at approximately 65% binding) to assess intra- and inter-assay variation. Intra-assay variation was below 14% for all plates and averaged 4%; inter-assay variation was below 12%. Baseline samples were diluted 1:20 and stress-induced samples were diluted 1:100. All assays were run at the end of each sampling year in one run except in 2016-2017. These samples were assayed in two groups, one in December and one in March.

Statistical analyses

All statistical analyses used SPSS 24. Linear Mixed Models (LMM) were used to determine whether: 1) baseline CORT levels or condition index were affected by hunting (i.e., were levels different during hunting season as opposed to when there is no hunting) and 2) the duration of the hunting season affected either of these two variables. Thus, we ran two analyses for each dependent variable (CORT and BCI). A separate analysis was necessary to understand the effects of the duration of hunting season because we only had duration data from birds sampled during hunting season (one of the four sampling periods). Thus, we could simplify the model by removing factors for this analysis. LMMs allowed us to evaluate candidate models that might have affected baseline CORT levels and condition index using Akaike's Information Criterion for small sample sizes (AICc; see below). All fixed factors and covariates included in our analyses can be found in Table 1. The first two LMMs determined whether either baseline CORT levels or BCI varied with hunted or not, sex, location, sample method, year, annual cycle stage, MinTemp, MaxTemp, BCI (for the baseline CORT dependent model), or baseline CORT (for the BCI dependent model). The second two LMMs determined if either baseline CORT or BCI varied in hunted Mallards with duration of hunting or the same covariates listed above with the exception of sample method and annual cycle stage. Time-of-day was also included in both baseline CORT models to control for possible circadian variation in CORT levels (Breuner et al., 1999). Less than three minute or lethal take plasma CORT samples with values above 10 ng/ml were considered stress induced and were not included in analyses (unpublished Mallard stress response data). Year and location were included in the analyses to control for known and unknown effects across years and locations, including climate variations, food availability, and habitat differences (Schoech et al., 2009). Baseline CORT and condition index values were not normally distributed and were transformed. Baseline CORT was log- transformed and condition index was square root transformed. Model assumptions of normality, collinearity, linearity, and homoscedasticity were confirmed in all cases.

Model selection was based on the lowest AICc value that indicates which variable or combination of variables best explained the variation in the model (Wilcoxen et al., 2011; Small and Schoech, 2015). We calculated AICc values for all variable combinations and then calculated the difference in AICc from that model to the best model (ΔAICc). All models were then ranked based on ΔAICc. Models with ΔAICc < 10 are shown and only models with ΔAICc values < 2 offer much information (Burnham and Anderson, 2002). Akaike weights (*wi*) were then calculated for each model and show the likelihood that model is the best model within the models tested.

To examine if differences in baseline CORT between sample methods could be due to sampling time an ANOVA with a Fisher's Least Squares Difference (LSD) post hoc tests was used. Sampling time was grouped by time to first bleed from capture as '0' (lethal take), 2, 2.5, and 3 minutes. For those cases in which the initial LMM revealed a significant effect of a factor or a covariate, we used regression analyses and ANOVAs to better understand the direction of the effect or the nature of the among group differences. LSD post hoc tests were used to better understand relationships among groups.

Table 1. List of variables used in this study.

Variables in Analyses	
Hunted or Not$_f$	During hunting season or not
Sex$_f$	Male or Female
Location$_f$	Sample location
Sample Method$_f$	Net trapped or lethal take
Year$_c$	Year of study (1-4)
Annual Cycle Stage$_c$	Grouped into fall pre-migration, migration pre-hunting season, migration/wintering during hunting season, spring pre-migration
Time-of-Day$_c$	Time-of-day sample was taken
MinTemp$_c$	Low temperature on day sample collected
MaxTemp$_c$	High temperature on day sample collected
BCI$_c$	Body condition index based on mass corrected for size (Sqrt-transformed)
BaseCORT$_c$	Baseline plasma CORT (log-transformed)
Duration Hunt$_c$	Number of days since the first day of hunting season

All main effects were included in analyses. $_f$ denotes fixed factors and $_c$ denotes covariates in the models.

RESULTS

Effects of hunting season and covariates on baseline CORT and BCI.

Baseline CORT levels were significantly higher in live individuals (netted) than they were in dead Mallards (hunter harvested; ($F_{1,116} = 6.809$, $p = 0.010$; Table 2; Fig. 1A). Further exploration found that 'baseline' CORT levels of netted birds increased with time ($F_{1,118} = 2.99$, $p = 0.034$; Fig. 1B), as birds sampled at time 0 had significantly lower CORT levels than birds sampled at 2.5 minutes ($p = 0.019$).

Table 2. Akaike's information criterion for small sample-sizes (AICc) to select variables (see above for rationale for variable consideration) for model inclusion in our explorations of Mallard baseline CORT and BCI across the fall and winter.

	Model	**K**	**AICc**	**ΔAICc**	**w**
Baseline CORT	Sample Method	**3**	**77.704**	**0.000**	**0.616**
	Sample Method + Year	4	79.607	1.903	0.238
	Sample Method + Year + Hunted or Not	5	80.611	2.907	0.144
	Sample Method + Year + Hunted or Not + MinTemp + MaxTemp + Time-of-Day + Annual Cycle Stage + Location + Sex + + BCI	15	120.173	42.47	0.000
Condition Index	Sample Method + Year + Hunted or Not + Annual Cycle Stage + Location + Sex + MinTemp + MaxTemp + BaseCORT	**14**	**765.745**	**0.000**	**0.504**
	Sample Method + Year + Hunted or Not + Annual Cycle Stage + Location + Sex + Tmax + BaseCORT	13	766.538	0.793	0.339
	Sample Method + Year + Hunted or Not + Annual Cycle Stage + Location + Sex + Tmin + Tmax	13	769.455	3.710	0.079
	Sample Method + Hunted or Not + Annual Cycle Stage + Location + Sex + Tmin + Tmax + BaseCORT	13	769.848	4.103	0.065
	Sample Method + Year + Hunted or Not + Annual Cycle Stage + Location + Sex + Tmin + Tmax + baseCORT	13	773.254	7.509	0.012

Shown AICc values: the number of parameters (K), the difference between each model and the best model (ΔAICc), and the Akaike weight, or the likelihood of that model being the best model (*wi*). The AICc values were derived from linear mixed models with baseline CORT or BCI as the dependent variable. The best model (lowest AICc), models with ΔAICc < 10, and the original model with all variables are displayed.

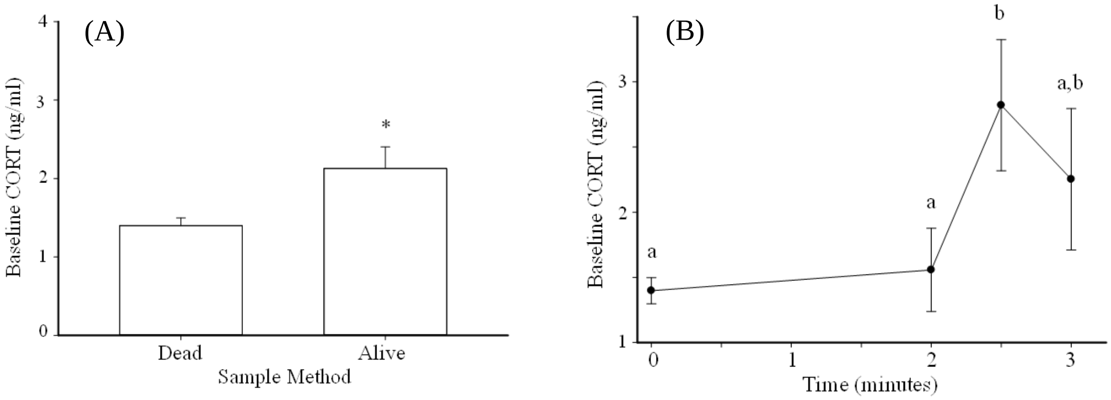

Figure 1. Effects of sample method on baseline CORT levels. (A) Mean (± SE) baseline CORT level of shot birds was significantly lower than that of netted birds ($p = 0.01$). Dead or alive refers to the status of the bird at time blood sample was taken. (B) Mean (± SE) baseline CORT levels across sampling times. Different letters indicate significant differences among time points.

Body condition varied significantly with sample method ($F_{1,105} = 6.98$, $p = 0.0095$), location ($F_{4,105} = 6.59$, $p = 0.0001$), hunted or not ($F_{1,105} = 16.19$, $p = 0.0001$), annual cycle stage ($F_{1,105} = 117.92$, $p = 0.0001$), sex ($F_{1,105} = 4.18$, $p = 0.043$), and MaxTemp, ($F_{1,105} = 21.37$, $p = 0.0001$: Table 2; Fig. 2).

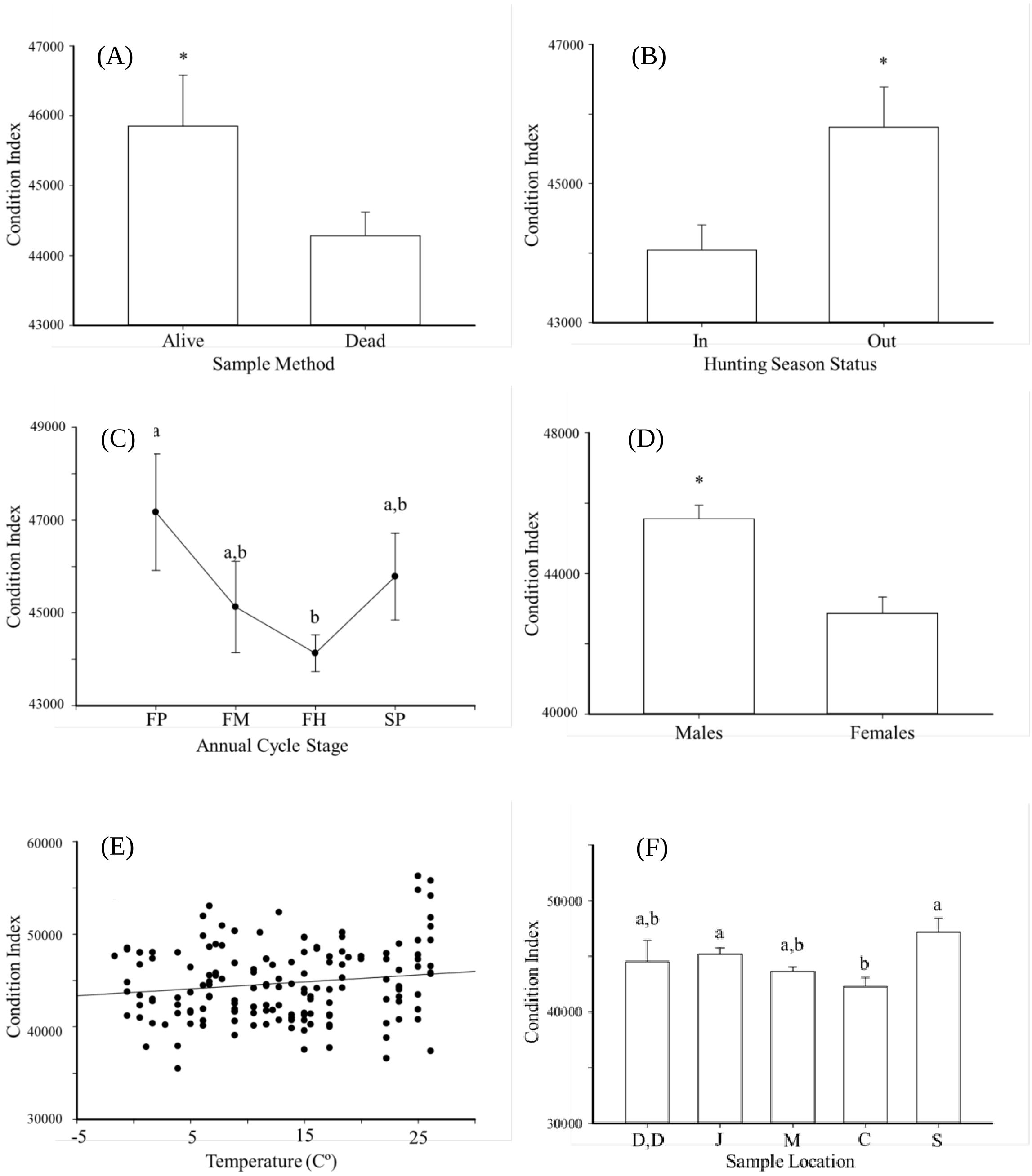

Figure 2. Effects of sample method (A), Hunted or Not (B), Annual Cycle Stage (C), sex (D), MaxTemp (E), and location (F) on body condition index. Body condition indices (mean ± SE) of Mallards: A) that were netted versus lethally collected; B) sampled during either the hunting season or the off season; C) across the annual cycles during the fall pre-migratory (FP), fall migratory (FM), fall/winter migratory during hunting season (FH), and spring pre-migration (SP) stages; D) by sex; E) against the high temperature (MaxTemp) on the day they were sampled; and F) across sample locations in (D,D) Drew and Desha, (J) Jackson, (M) Monroe, (C) Crittenden, and (S) J. Clark Salyer NWR. Sample locations are reported as county of sampling or refuge for ND. * or different letters indicate significant differences between groups ($p < 0.05$).

Effects of hunting duration and covariates on baseline CORT and BCI

Among the subset of Mallards that was sampled during the hunting season, there was no significant effect of the duration of the season on baseline CORT levels. Of all the variables included in the original model, year explained the most variation, although this effect fell short of statistical significance (p = 0.090; Table 3). Body condition among the same subset of hunted Mallards varied significantly with location, sex, and MaxTemp ($F_{1,83}$ = 9.467, p = 0.003; $F_{1,83}$ = 15.200, p = 0.000; $F_{1,83}$ =17.458, p = 0.000; Table 3; Fig. 3).

Table 3. Akaike's information criterion for small sample-sizes (AICc) to select variables (see above for rationale for variable consideration) for model inclusion in our explorations of Mallard baseline CORT and BCI during the hunting season.

	Model	**K**	**AICc**	**ΔAICc**	**w**
Baseline CORT	Year	**2**	**71.632**	**0.000**	**0.874**
	Year + Tmin	3	80.18	8.552	0.007
	Sex + Location + time of day + Year + CI + Tmin + Tmax + Duration Hunt	10	115.70	44.072	0.000
Condition Index	sex + Location + Year + Tmax + BaseCORT	**7**	**593.086**	**0.000**	**0.37**
	Sex + Location + Year + Tmax + Duration Hunt + BaseCORT	8	594.19	1.099	0.21
	sex + Location + Year + Tmin + Tmax + BaseCORT	8	594.27	1.19	0.20
	Sex + Location + Year + Tmin + Tmax + Duration Hunt + BaseCORT	9	596.54	3.45	0.066
	Sex + Location + Year + Tmax	6	596.57	3.48	0.065
	Sex + Location + Year + Tmax + Duration Hunt	7	597.67	4.58	0.037
	Sex + Location + Tmax + BaseCORT	6	598.514	5.428	0.024
	Sex + Location + Year + Tmin + Tmax + Duration Hunt	8	600.050	6.964	0.011
	Sex + Location + Tmin + Tmax + Duration Hunt + BaseCORT	8	600.563	7.477	0.009

Shown AICc values: the number of parameters (K), the difference between each model and the best model (ΔAICc), and the Akaike weight, or the likelihood of that model being the best model (*wi*). The AICc values were derived from linear mixed models with baseline CORT or BCI as the dependent variable. The best model (lowest AICc), models with ΔAICc < 10, and the original model with all variables are displayed.

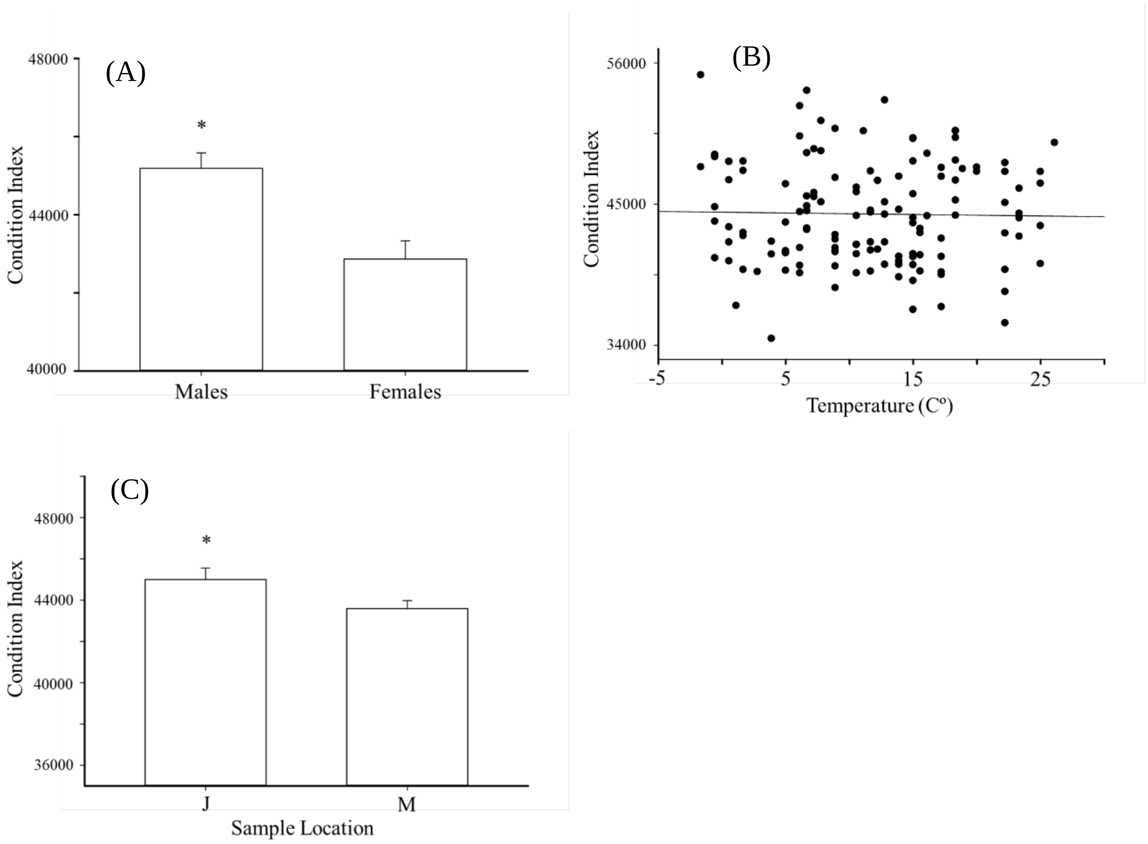

Figure 3. Effects of sex (A), MaxTemp (B), and location (C) on body condition index of Mallards sampled during hunting season. Mean (± SE) body condition index of Mallards: A) by sexes; B) against the high temperature (MaxTemp); and C) by sampling location (labeled same as figure 1F). * indicates significant difference between groups ($p < 0.05$).

DISCUSSION

The results indicate that hunting season was a factor that contributed to body condition in Mallards. During the hunting season BCI was lower as compared to the non-hunting season. The decrease in body condition due to hunting season was in addition to an overall larger decrease that occurred across the fall and winter (Fig. 2B and 2C). It's interesting to note that there was also an effect of sex, temperature, and location on BCI. Females had lower body condition indices than males. Body condition increased with warmer daily high temperatures except during hunting season and varied across sampling locations. We also found that baseline CORT and body condition differed between collection methods: birds caught in nets had higher baseline CORT levels and were in better body condition than lethally taken birds.

Effects of Hunting

Although there were no differences in baseline CORT levels in birds that were or were not exposed to hunting, there was a significant difference in body condition. Birds sampled during hunting season had a lower BCI than those sampled out of the hunting season. Mallard body condition also decreased through fall migration into the wintering period. The seasonal decrease in body condition is well documented and is the result of increased energetic demands and endogenous rhythms (Whyte and Bolen, 1984; Loesch et al., 1992; Haukos et al., 2001). The decrease in body condition due to hunting was in addition to the seasonal decrease as both were factors in the best model. Any further decrease beyond the seasonal decline could limit Mallards' ability to progress through annual cycle stages and ultimately affect reproduction (Heitmeyer, 1988b, 2006; Devries et al., 2008). Interestingly, the decrease in body condition was observed without an increase in baseline CORT, which could be the result of changes in behavior such as increased vigilance, irruptive behaviors, and decreased time spent foraging. It is encouraging to see body condition begin to recover quickly into the spring pre-migratory period. Thus, it is possible for Mallards to recover body condition through the spring given ample resources (Dubovsky et al., 1994). We speculate that the main factor contributing to the variation in body condition is the disturbance associated with hunting season. However, given that there was a concurrent effect of annual cycle stage upon body condition, caution is necessary and further analysis or study is needed to tease apart which of these two variables is most responsible and to strengthen this conclusion.

Given the prolonged exposure to hunting pressure as the season progresses, it might be expected to see an increase in CORT and decrement in body condition (Silverin, 1998; Scheuerlein et al., 2001; Cockrem and Silverin, 2002; Jones et al., 2016). However, this effect could be mitigated by changes in the stress response or behavior. Birds can combat the negative consequences of prolonged elevated CORT under circumstances in which the stress is predictable by minimizing CORT release in response to stressors (Wingfield et al., 1995; Romero et al., 1997, 1998; Wilson et Al., 2004, 2017; Romero, 2006;). For example, birds commonly dampen their stress response during times when elevated CORT may not be compatible with the increased demands of certain stages of the annual cycle (e.g., molt, reproduction, and migration; Wingfield et al. 1995). Additionally, repeated or chronic exposure to stressful stimuli can lead to an overall decrease in CORT levels due to either exhaustion or perhaps as a protective mechanism (see Cyr and Romero, 2007, 2009). Increases in CORT can also increase vigilance behaviors, making birds more wary and thereby able to avoid future stressful situations (Cockrem and Silverin, 2002, Jones et al., 2016). If this were the case and hunting pressure over the past century had caused a change in behavior due to increased wariness, one might expect to find shifts in migratory patterns or wintering population locations to avoid heavily hunted stressful areas (Cox and Afton, 1997, Madsen, 1998).

Effects of Seasonality and Temperature

Body condition also varied with annual cycle stage, temperature, and location (placed in the model to control for any unknown effects). BCI was highest in the fall pre-migratory period then decreased across the fall and winter but began to increase again in preparation for spring migration. Initial sampling was done in September on pre-migratory Mallards in ND. These birds had just completed their wing molt and were staging for fall migration. The study period ended when birds initiated spring migration at the end of February. This finding is in agreement with Whyte and Bolen (1984) and Haukos et al. (2001), who found an overall decrease in body mass, particularly in lipid stores, of Mallards across the fall and winter followed by an increase before spring migration. Increased energetic demands of migration, courtship, thermoregulation, or seasonal weight loss thought to facilitate impending long-distance flight, could explain the seasonal decrease in body condition. The pre-migration increase in body condition is likely in preparation for these demands (Brodsky and Weatherhead, 1985; Heitmeyer 1988b; Loesch et al., 1992).

Body condition increased as daily high temperatures increased across the study period. As temperatures increase during the fall and winter, resources are more available and energetic demands of thermoregulation are reduced (Legagneux et al., 2009). Cold temperatures can limit resources due to ice and warmer temperatures can initiate invertebrate hatches and vegetation growth (Wehrle et al., 1995; Manley et al., 2004). The small negative relationship between daily high temperatures and body condition during hunting season, opposite of the overall trend, could be an artifact due to sample method. Birds in poor body condition require more resources and may engage in more risk prone behaviors to find food. Thus, birds in poor body condition had a higher likelihood to be sampled (i.e. taken by hunters) on warm days when birds in good health likely spend less time in search of food.

Sampling Method Bias

Throughout this study, we observed a bias in body condition and baseline CORT levels between sampling methods. We had a difficult time netting birds during the hunting season as, (due to federal regulations) we were unable to use bait to attract Mallards to netting sites. To control for this and get a comparison between the sampling methods, as well as compare before, during, and after hunting season groups, we sampled a subset of birds pre- and post-hunting season that were lethally taken. These samples overlapped with many of our net trapped samples and this way we were able to control for annual cycle stage when comparing groups. Net trapped Mallards were in better body condition and had higher baseline CORT levels than those that were shot. Sampling bias in body condition between net trapped and hunter harvested birds has often been observed (Weatherhead and Greenwood, 1981; Hepp et al., 1986; Reinecke and Shaiffer, 1988). This was termed the Condition Bias Hypothesis which states that Mallards in poorer body condition are more susceptible to hunters. This bias could be due to many factors, although a common thread is the idea that birds in poor body condition are more likely to engage in risk prone behaviors (Reinecke and Shaiffer, 1988). It follows that the risk prone behaviors in combination with the gregarious nature of unpaired Mallards increase the odds of their being fatally attracted to a hunter's decoys. Of further consideration, Mallards in better body condition are at the top of the dominance hierarchy and will be the first to find a mate for the season (Heitmeyer, 1988b; Holmberg et al., 1989). Mallards begin courtship and pairing over the winter and many Mallards have found a mate by the end of December (Johnsguard, 1960; Johnson and Rohwer, 1998). This is midway through the hunting season and paired Mallards are reclusive and seek out thickets to strengthen their pair-bond and avoid competition for their mate. This avoidance behavior further decreases the likelihood that they are drawn to Mallard decoys. Conversely, Mallards in poorer condition driven by the urge to breed will seek out flocks of Mallards as they search for a mate throughout hunting season making them more susceptible to hunters.

The elevated baseline CORT levels in net trapped birds are most likely an artifact of sampling time. While net trapped birds were sampled between 1 and 2.5 min post capture, hunter harvested birds were sampled at a time 0. Thus, all birds sampled via hunter harvest had zero sampling stress, but birds that were net captured were exposed to from 1 to 2.5 min of capture stress before a blood sample could be obtained. This finding supports the hypothesis that 3 minutes may not be fast enough to determine a true baseline CORT measure (Romero and Reed, 2005; Small et al., 2017). A separate factor that might have contributed to the apparent bias in baseline CORT levels between netted and harvested Mallards is that netted birds were 'driven' by CORT-induced hyperphagia and were disproportionally attracted to the bait used for netting (see Wingfield et al., 1998).

Conclusion

To the best of our knowledge, this is the first study to examine the effects of hunting on the stress physiology of a migratory bird species. It is important to acknowledge that hunting season can have an effect on body condition, but more research is needed to determine how much these effects are in addition to seasonal decreases in body condition. This study begins to address how a wild population copes with a long-term anthropogenic stressor and may have particularly important impacts on how hunting seasons affect waterfowl, with this knowledge being used to establish better waterfowl management practices. This study also reveals discrepancies in CORT measures as a function of sampling method indicating that caution should be used regarding the interpretation of baseline CORT even within the generally accepted 3 minute window (see Small et al., 2017).

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CHAPTER 4: MALLARDS (*ANAS PLATYRHYNCHOS)* REGULATE STRESS RESPONSIVENESS ACCORDING TO ENERGETIC DEMANDS DURING THE FALL AND WINTER

Introduction

How birds cope with changes in their environment directly affects their fitness. This is particularly important for game birds that endure changes in their environment across their annual cycle, while also facing a major anthropogenic stressor (i.e., hunting season). They exhibit seasonal adjustments in physiology and behavior to cope with changes in energetic demands and resource availability.

It is well known that glucocorticoids play a major role in meeting energetic demands (Romero et al., 2009; Sapolsky et al., 2000; Siegel, 1980; Wingfield et al., 1998). The major avian glucocorticoid is corticosterone (CORT) (Siegel, 1980). An increase in CORT release from the adrenal cortex following activation of the hypothalamic-pituitary-adrenal (HPA) axis is generally detectable within two to three minutes of a stressful encounter (Romero & Romero, 2002; Romero & Reed, 2005; Small et al., 2017). Elevated plasma CORT levels affect a suite of behavioral and physiological changes that aid the management and survival of a stressful encounter (Wingfield et al., 1998). In birds, the effects of elevated CORT include increased blood glucose levels via gluconeogenesis (through protein and fat catabolism), suppression of reproductive behaviors, down regulation of the immune system, increased foraging behavior, promotion of irruptive behavior during the day, and reduced metabolic rate at night (with the exception of times of nocturnal migration) (Sapolsky et al., 2000; Wingfield et al., 1998). These effects are beneficial over a short duration (*acute*; i.e., minutes to hours) but if prolonged (*chronic*; i.e., weeks to months) can result in decrements in body condition, social standing, and cognitive performance, as well as delayed onset (or the abandonment) of reproduction (Boonstra, 2004; Schoech et al., 2009, 2011; Siegel, 1980; Wingfield et al., 1998). Thus, it is important for birds to minimize prolonged exposure to elevated CORT, especially during times of the year that have increased energetic demands associated with annual cycle stages.

The annual cycles of birds can be relatively simple to quite complex, and that of the Mallard (*Anas platyrhynchos*) falls on the complex side (Bluhm, 1988). During the fall and winter, Mallards complete their pre-basic molt, prepare for and undergo fall migration, undertake the pre-alternate molt, begin courtship and pairing, and prepare for spring migration (Baldassarre, 2014; Bluhm, 1988; Dugger, 1997; Heitmeyer, 1988a; 1988b; 2006; Richardson and Kaminski, 1992; see Fig.1). All of the preceding, include energetic demands.

Birds can mitigate potential effects of chronic stress by altering how they respond to a stressor (Romero et al., 1998). Many birds respond to predictable energetically demanding times by minimizing CORT exposure (Romero et al., 1997, 1998; Romero, 2006; Wilson et al., 2004, 2017; Wingfield et al., 1995). It is not uncommon for birds to dampen their stress response during times when elevated CORT may not be compatible with the increased demands of certain stages of the annual cycle (e.g., molt, reproduction, and migration; Wingfield et al. 1995). Additionally, repeated or chronic exposure to stressful stimuli can lead to an overall decrease in CORT levels due to either exhaustion or perhaps as a protective mechanism (see Cyr and Romero, 2007, 2009).

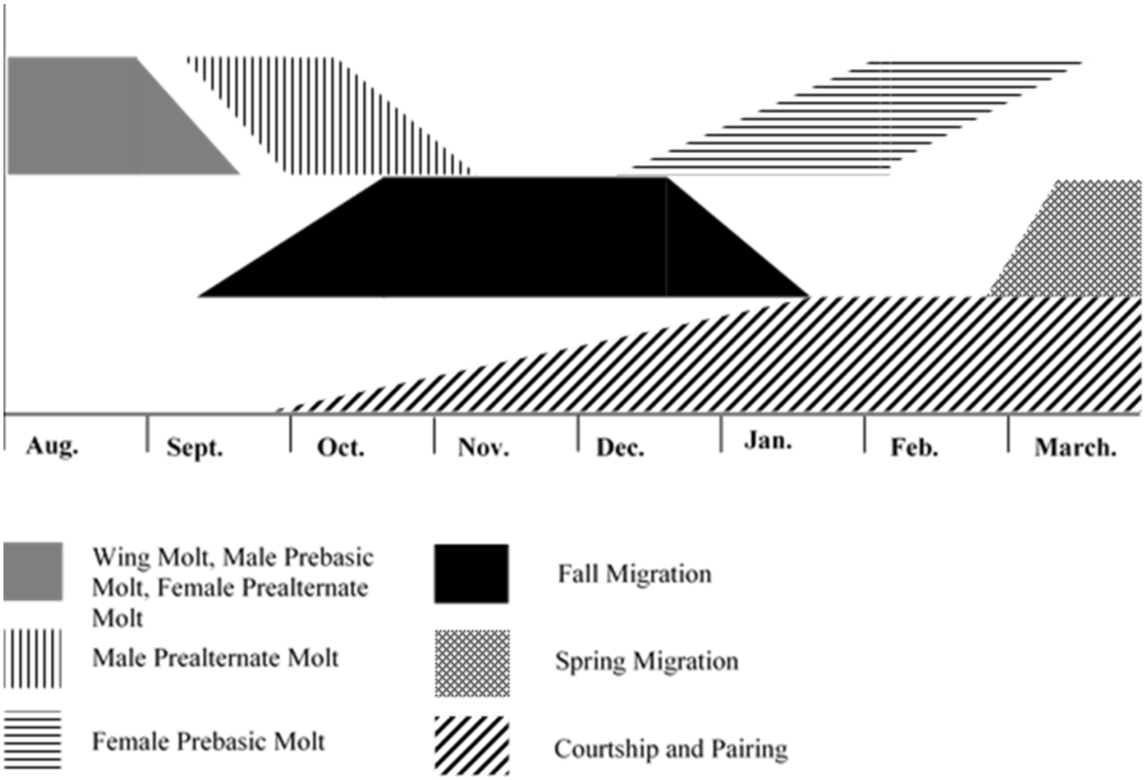

Fig. 1. Major life history stages of Mallards during the fall and winter study period. Figure shows the varying degrees of overlap of life history stages during sample collection (compiled from Baldassarre, 2014).

Recent results from Wilson et al. (2017) and Henson et al. (in prep) revealed no differences in baseline CORT levels (collected within 3 min of capture/handling) across fall and winter in blue-winged teal (*A. discors*) or Mallards. These findings are rather surprising given that this is a time when energetic demands and stressful encounters should be amplified due to the demands of the annual cycle (see Fig.1) and the concurrent waterfowl hunting season. Thus, it is possible that both species are dampening their HPA axis responsiveness to ameliorate the negative effects of elevated CORT during this time. The aim of this study was to determine whether HPA axis responsiveness in Mallards differs across the fall and winter portion of the annual cycle. We hypothesize that Mallards will respond differently to a standardized stressor across annual cycles.

Methods

Study Populations

Mallards were sampled in November, January, and February in eastern Arkansas in Jackson, Crittenden, Drew, and Desha counties. Samples were collected during fall migration/wintering (November through January) and through pre-spring migration (February and March) in flooded bottomland hardwood forest and flooded rice and soybean agricultural fields over four years (2013-2017). Samples from the fall pre-migration period were collected from the Souris River valley at J. Clark Salyer National Wildlife Refuge, North Dakota in September of 2016.

Data Collection

Mallards were captured using mist nets, rocket nets, and net launcher (Coda Netgun; Coda Enterprises, Mesa, AZ) (n=41, Sept = 23, Nov/Jan = 5, Feb = 13). Samples were collected during three periods: fall pre-migration (September 16 - 21; n = 23), migration /wintering (November 11 - January 31; n = 5), and spring pre-migration (February 15 - 20; n = 13). Netting Mallards during hunting season (mid-November – last week of January in Arkansas) proved difficult due to federal regulations that prohibited the use of bait. Thus, our sample size for the November - January sample period is small but seemingly sufficient as is reflected by the limited variance among these samples (see Fig. 2).

Mallards were subjected to a standardized capture and handling stress protocol that simulates an acute stressor (see Wingfield et al., 1992). A blood sample was taken within 2.5 minutes of capture for baseline samples. Then, ducks were placed in a loosely woven bag and sampled again at 15, 30, and 60 minutes to provide a stress response curve for each individual. An integrated stress-induced CORT value (area under the curve minus the baseline; corrected integrated CORT; see Cockrem and Silverin, 2002) was calculated for each stress response curve to determine the total stress-induced CORT individuals were exposed to over the course of the capture and handling protocol. Blood samples were taken from the brachial vein (140 – 280 µl) using a 25 gauge needle and heparinized capillary tubes. Samples were kept on ice (2-5 hr) until they could be centrifuged (11,500 rpm for 10 minutes) and the plasma drawn off. Plasma was stored at -20˚C until assay.

Following sample collection and before release, birds received a USGS leg band and were sexed based on plumage. Mass and wing cord length were also recorded to create a body condition index (BCI) using the methods of Peig and Green (2009, 2010). All research was done under USGS banding permit #23254, USFWS scientific collection permit #MB49809B-1, AGFC scientific collection permit #011320172, and ND scientific collection permit #GNF04121011.

Sample Analyses

Plasma CORT (total CORT; see Schoech et al., 2013) was quantified using 10-15 µl of plasma. Samples were assayed with a CORT ELISA from Cayman Chemical, Ann

Arbor, MI (Cat No. 500655), which has been validated in our lab for use in waterfowl (Wilson et al., 2017; Small and Schoech, 2015). Each sample was run in duplicate and a standard curve from 5000 pg/ml to 8.2 pg/ml was generated using a known concentration of CORT. Each plate contained internal controls (high IC 600 pg/ml at approximately 20% binding; low IC 60 pg/ml at approximately 65% binding) to assess intra- and inter-assay variation. Intra-assay variation was below 14% for all plates and averaged 4%; inter-assay variation was 11%. Baseline samples were diluted 1:20 and stress-induced samples were diluted 1:100. All assays were run at the end of each sampling year in one run except in 2016-2017. These samples were assayed in two groups, one in December and one in March.

Statistical analyses

HPA axis responses among sampling periods were analyzed with a repeated-measures ANOVA and, in cases where a significant main effect was detected, further exploration was made with a Fisher's least-square difference (LSD) post hoc test. Area under the stress curve minus the baseline (corrected integrated CORT) was used as a secondary measure for stress responsiveness. Corrected integrated CORT and BCI among sampling periods were analyzed by ANOVA followed with LSD post hoc test. BCI was used a covariate in the corrected integrated CORT analyses. Initially, sex was used as a factor in both analyses and did not affect either stress-induced CORT measure ($p > 0.6$ for both). Thus, due to small sample sizes, males and females were combined for all analyses. All statistical analyses used SPSS 24.

Results

There was a significant difference in HPA axis responses among sampling periods (repeated-measures ANOVA; $F_{6,56} = 7.93$, $p < 0.0001$; Fig. 2). HPA axis responsiveness differed significantly among the three periods ($p \leq 0.002$ for all; see Fig. 2), with lowest levels during the fall migration/wintering period, intermediate levels during the fall pre-migratory period, and highest during the spring pre-migratory period. Corrected integrated CORT also varied as a function of sampling period (ANOVA; $F_{2,42} = 35.00$, $p = 0.000$; Fig. 2B), and the pattern did not differ from that observed for the stress response (LSD $p \leq 0.005$ for all; Fig. 2B). Body condition did not vary across the sampling period and corrected integrated CORT did not vary as a function of body condition ($p \geq 0.3$ for both).

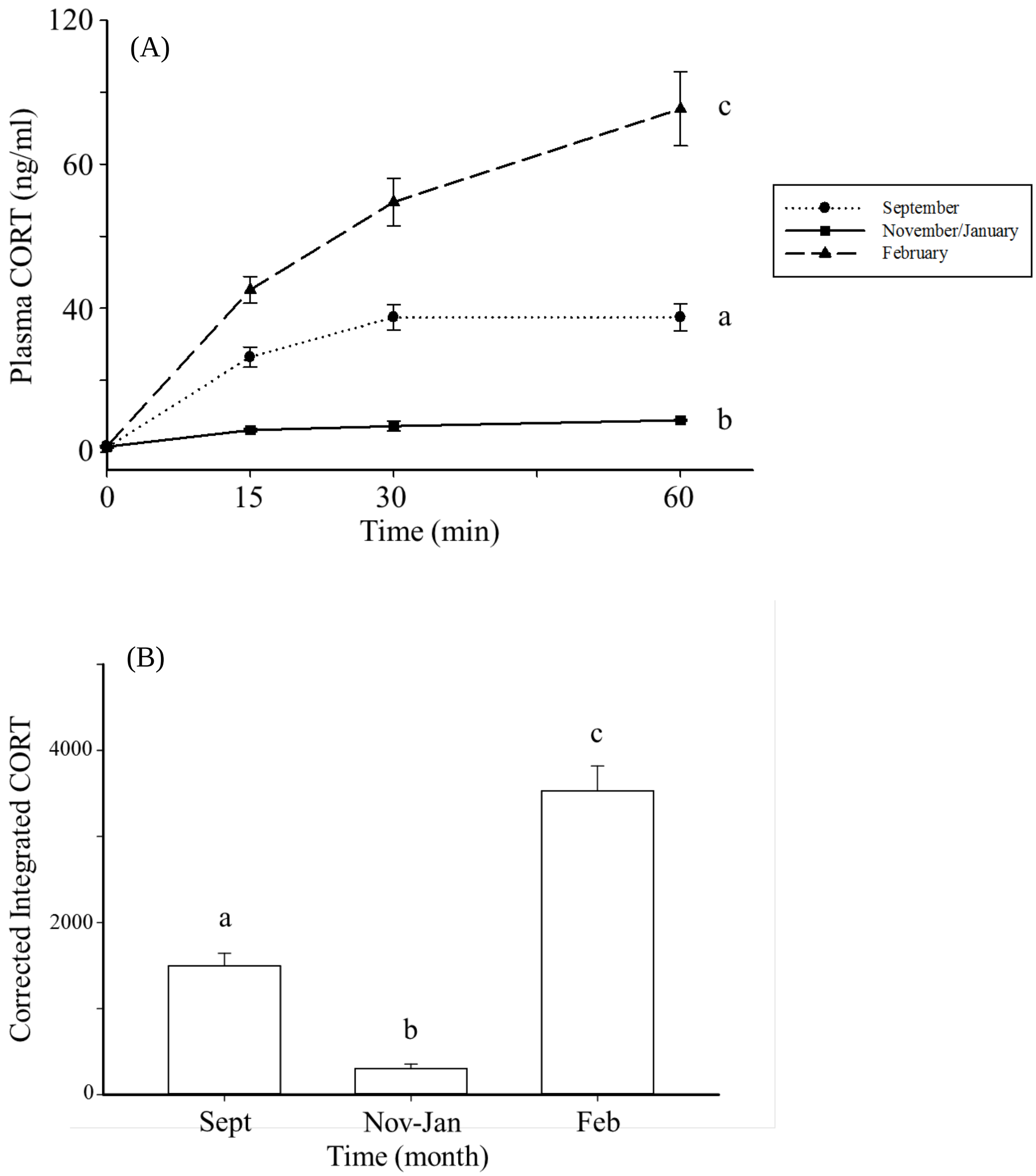

Figure 2. Stress responsiveness across sampling period. (A) Mean (± SE) HPA axis responses to capture and handling varied among sampling periods in the fall and winter. Different letters indicate significance differences among periods $p \leq 0.002$. (B) Effects of sample month on corrected integrated CORT. Mean (± SE) corrected integrated CORT. Different letters indicate significant differences among time points $p \leq 0.005$.

Discussion

It is evident that Mallards modify their CORT response seasonally, likely in response to differing demands across the annual cycle. HPA axis responsiveness varies across all three sampling periods of this study, in marked contrast to baseline CORT levels assessed across these same periods (Henson et al., in prep). HPA axis responsiveness was lowest during the November through January period which coincides with fall migration and overlaps to some degree with both male and female prealternate molts (see Fig.1). Mallard's regularly undergo southward movement well into January and have migrated south as late as mid-March due to cold weather events, thus 'fall migration' is somewhat of a misnomer (Baldassarre, 2014). Stress responsiveness was highest in February when Mallards are wintering and preparing for spring migration. Interestingly and paradoxically, this period of highest responsiveness co-occurs with female prebasic molt. This led us to compare male and female responsiveness during February, which showed no significant difference (*p*=0.7). The level of stress responsiveness during the fall pre-migratory period was intermediate to, and significantly different from, the other two periods (Fig. 2 and 3). Mallards sampled at this time (15 – 21 Sept.) were post wing molt and completing a body molt.

Many bird species exhibit reduced HPA axis responsiveness during the fall and winter in comparison to those of the breeding season (Astheimer et al. 1994,1995; Romero et al., 1997; Romero, 2006; Wilson et al., 2004, 2017). HPA axis responsiveness is reduced during migration in blue-winged teal Wilson et al. (2017). However, unlike their blue-winged teal cousins that are obligate migrators (i.e., exhibit a temporally fixed, photoperiod-driven migration that varies little year-to-year), Mallards are facultative migrators for which resource availability dictates the timing and latitudinal extent of migration (Hepp et al., 1986). Thus, it appears that regardless of migration strategy, waterfowl dampen their HPA axis responsiveness during fall migration; however, information regarding additional species is needed to support this conclusion.

Our findings, that the lowest degree of CORT responsiveness occurs during the fall migration/wintering period is in agreement with previous findings of dampened CORT responses during times of extreme energetic demand (e.g., migration). Further, given the evidence of HPA axis dampening during molt observed in other species (e.g., house sparrows (*Passer domesticus*; Romero, 2006), white-crowned sparrows (*Zonotrichia leucophrys*; Astheimer et al., 1995; Romero et al., 1997, 2005) , lapland longspurs (*Calcarius lapponicus*; Romero et al., 1998), and european starlings (*Sturnus vulgaris*; Cyr et al., 2008; Romero et al., 2005), samples collected during the fall migration/wintering period overlap of a portion of the male prealternate molt and the female prebasic molt (Baldassarre, 2014; Bluhm, 1988, Heitmeyer, 1988b; Fig. 1). Which, may contribute to our finding of a markedly dampened stress responsiveness during this period.

One of the surprising results from this study was the particularly high HPA axis responsiveness during the spring pre-migratory period. Most species studied exhibit a dampened stress response throughout fall and winter with periods of high HPA axis response restricted to the breeding season (Astheimer et al., 1994, 1995; Romero et al., 1997; Romero, 2006; Wilson et al., 2017). Mallards begin to nest in spring after completing migration, but courtship and pairing occurs during fall and winter, with 90 - 95% paired by January (Johnson and Rohwer, 1998). Increased HPA axis responsiveness in waterfowl may correlate with the completion of pair formation and courtship rather than the onset of nesting. Thus, the CORT responsiveness we measured during the spring pre-migratory period is similar to the values in territorial male blue-winged teal (sampled in May), a species that completes pair formation in April/May (Bluhm, 1988; Wilson et al. 2017).

Birds in poor body condition often exhibit a reduction in adrenocorticoid responsiveness, presumably because their poor condition renders them incapable of producing a robust response (Jenni et al., 2000; Mizrahi et al., 2001, but see Perfito et al., 2002). Our data do not support this hypothesis and indicate no effect of BCI on HPA axis response. Additionally, there was no significant difference in BCI across sampling periods, thus birds with similar BCI exhibited strikingly different HPA axis responsiveness dependent on annual cycle stage.

Our results indicate that exposure to CORT is lowest during the fall migration/wintering period, presumably a time of increased energetic demand. The highest CORT responsiveness occurred during the spring pre-migratory period, at the completion of pair formation. The lowest CORT responsiveness co-occurs with the annual cycle stages of migration and molt, both of which place an increased demand on protein and lipid reserves. Due to the overlap of naturally occurring challenges, such as inclement weather, migration, and molt with the anthropogenic challenge of the hunting season, it is difficult to assess which is the primary factor affecting HPA responsiveness or if this is an evolutionary response to the combination of factors. Alternatively, the low CORT responsiveness may reflect exhaustion of the HPA axis due to repeated stressful encounters. Additional studies that measure an individual’s degree of molt, as well as consider wild birds in a non-hunted environment are needed to better determine the relative importance of these factors. Similarly, to understand the relationship between HPA axis activity and the various stages of the annual cycle, Mallards need to be studied throughout the year.

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CHAPTER 5: CONCLUSION

From the results of Study 1 (see Chapter 2), I determined that all three measures of condition (i.e., TRIG, BCI, and body mass) were positively correlated with subcutaneous fat and, whereas BCI and TRIG combined explained 35% of the variation, TRIG alone were a poor predictor that explained about 7%. These results indicate that while TRIG were correlated with fat deposits, they alone are not a practical tool for estimating body fat. I determined in Study 2 (Chapter 3), that baseline CORT was not affected by hunting but body condition was relatively poor in Mallards sampled during hunting season. Body condition was negatively affected by hunting pressure and also was poorer as the season progressed from fall through the winter. Interestingly, birds caught in nets had higher baseline CORT levels and were in better body condition than lethally taken birds. These results support the hypothesis that hunting disturbance affects the body condition of birds, and make it clear that sampling method should be considered when measuring baseline CORT or body condition in Mallards. In our final Study (Chapter 4), that CORT response was reduced during fall migration and increased during the late winter when Mallards complete pair formation for the upcoming breeding season. However, body condition and CORT responsiveness were not correlated. These results are similar to other studies that found a reduced CORT response during migration and molt, and increased responsiveness associated with breeding behaviors.

Taken together these studies indicate that BCI was the best indicator of Mallard overall body condition (fat deposits) and that BCI, but not baseline CORT, varied across the fall and winter portion of the annual cycle depending on the pressures and demands associated with that time of year. I also found that Mallards dampen their response to a stressor during periods of increased energetic demands, such as migration. This offers further support for the idea that birds do so to minimize the negative effects of elevated CORT on body condition.

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17 April 2013

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Dr. Sims:

The Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas-Monticello has approved your research proposal entitled, "Impacts of hunting and life-history stages on stress physiology of wintering mallards (*Anas platyrhynchos*)." Your research permit number is 04172013. Significant changes to your research protocols require prior approval by the IACUC.

Your collection permit from the Arkansas Game and Fish Commission (AGFC) expires 14 January 2014 and your Federal Bird Banding Permit from the US Fish and Wildlife Service (USFWS) expires 30 September 2013. Your proposal, however, indicates an October 2013 start date and October 2016 completion date for your research. I am aware that the AGFC and the USFWS issues their research and bird banding permits annually. To maintain a valid IACUC research permit throughout the duration of your research, you must renew your research permits annually. When you obtain your renewed permits, please send me a copy of each so that I can place them in your file. It is important that you maintain valid AGFC research and USFWS bird banding permits throughout the duration of your research. Your proposal and a copy of this approval letter will be filed with the Office of the Vice Chancellor for Academic Affairs.

The IACUC wishes you great success in your research.

Sincerely,

Don White, Jr., Ph.D., CWB®
James M. White Professor of Wildlife Ecology
Chair, the Institutional Animal Care and Use Committee

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16 November 2015

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Dr. Sims:

The Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas-Monticello has approved your research proposal entitled, "Impacts of hunting and life-history stages on stress physiology of wintering mallards (*Anas platyrhynchos*)." Your research permit number is 11162013A. Significant changes to your research protocols require prior approval by the IACUC.

Please remember that it is your responsibility to maintain valid Arkansas Game and Fish Commission research and US Fish and Wildlife Service bird banding permits throughout the duration of your research project. Your proposal and a copy of this approval letter has been filed with the Office of the Vice Chancellor for Academic Affairs.

The IACUC wishes you great success in your research.

Sincerely,

Don White, Jr., Ph.D., CWB®
Wildlife Ecologist and James M. White Professor of Wildlife Ecology
Chair, the Institutional Animal Care and Use Committee