

# **Energetic costs of aggression in the Northern Cardinal**

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## **Abstract**

There is a lack of work studying territorial aggression during the nonbreeding season, especially in a natural system. There has been some evidence to suggest that the steroid hormone precursor, dehydroepiandrosterone (DHEA) may regulate male aggression during the nonbreeding season, rather than testosterone. This study aimed to investigate the effects of energy status (through food supplementation) on nonbreeding aggression in the Northern Cardinal and add to the pre-existing evidence of a DHEA-mediated mechanism of aggression. It was hypothesized that if aggression was maintained for an extended period of time, metabolite levels would favor energy usage (more glycerol and less triglycerides) compared to a bird who has exhibited aggression for only a short period of time. Furthermore, birds with greater food security (i.e., FS birds) will exhibit more aggression, and hence more DHEA and less corticosterone (CORT), while expending more energy than CON birds with less food security. However, the results of the study were inconclusive. While metabolite concentrations did not show a significant difference between treatment groups, it did appear that CORT was slightly lower in food supplemented birds and DHEA was higher and increased more drastically during capture than control birds. However, statistical analyses found that none of these difference observed from graphical representation of the data were significant. It was concluded that this can be attributed to a very small sample size and large individual variance. Therefore, we could not conclude with certainty that food-supplementation affected aggression or whether aggression was regulated by DHEA.

## Introduction

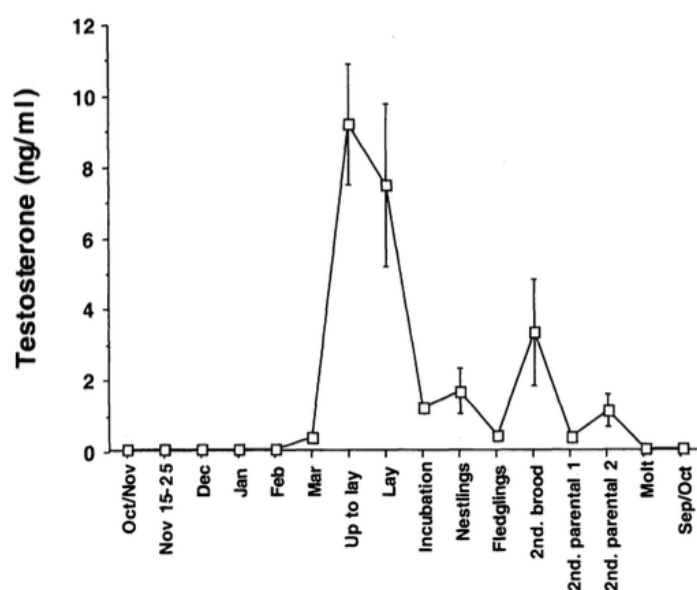
During the breeding season, males of many animal species establish and actively defend territories from conspecific intruders. Males primarily establish such territories in order to secure resources, such as food and shelter, for breeding and the provisioning of offspring. The territory also functions in mate guarding of the resident male's female partner, and when another male intrudes on the territory, the resident male will use aggressive behavior to drive it away and prevent the intruder from possibly mating with the female (Beletsky & Orians, 1989; Beletsky & Orians, 1987). There are several distinctive behaviors associated with territoriality. In songbirds, these behaviors can include song (solitary, or duets with a female), display flights, and bill wiping (Jawor, 2007; DeVries et al., 2011), and the ease of observation of these behaviors make birds a popular model for research on territoriality. Most of the information we have regarding territorial behavior and the underlying physiological mechanisms comes from studies conducted during the breeding season, where the territories function to attract females rather than to primarily secure food resources. Here, testosterone produced by the testes is the steroid hormone that is primarily responsible for eliciting territorial aggression during the breeding season (Jawor, 2007; Chiver et al., 2014). However, some species exhibit the same suite of behaviors in the nonbreeding season when testosterone levels are very low, and often undetectable, suggesting an alternative mechanism may regulate aggression outside of the breeding season (Wingfield et al., 1990; Simpson, 2001; Jawor, 2007).

Some recent research has suggested that dehydroepiandrosterone (DHEA), a steroid precursor that can be converted to testosterone with the appropriate enzymes, plays a significant role in the regulation of nonbreeding aggressive behavior (Soma, 2008).

Furthermore, DHEA is influenced by an individual's energy status (Patel and Katyare, 2006). Nonbreeding territories may function to secure scarce food resources rather than mates, and DHEA may provide a possible link between food availability and nonbreeding territoriality. A lack of available food can also induce stress, which can influence nonbreeding aggression by increasing levels of the stress steroid, corticosterone (CORT) which may drain the energy stores that are necessary to sustain aggressive behavior. This thesis research explores how energetic status influences territorial aggression and its associated steroids (testosterone, CORT, and DHEA) through a food supplementation experiment in the highly-territorial Northern Cardinal (*Cardinalis cardinalis*).

### I. Testosterone

Testosterone is a steroid sex hormone classified as an androgen and is produced primarily by the gonads; the testes in males and to a lesser extent the ovaries in females, where it is



**Figure 1.** The pattern of testosterone secretion of a typical free-living songbird, the rufous song sparrow (taken from Wingfield & Soma 2002).

metabolized into estrogen by the enzyme aromatase (Simpson, 2001; Soma, 2006). There is also some testosterone production by the adrenal glands, which are primarily responsible for releasing hormones in response to stress (Soma, 2006). In order to elicit an aggressive response to stimuli in an animal, testosterone binds to neural androgen receptors

after prior conversion to 5-alpha-dihydrotestosterone (DHT) by a reductase enzyme or more often by conversion to estradiol (E<sub>2</sub>) by the aromatase, to bind to neural estrogen receptors (Simpson, 2001). Generally, testosterone levels peak significantly during the breeding season, and are then reduced to nearly undetectable levels during the nonbreeding season when the testes shrink significantly. This seasonal pattern has been demonstrated in the rufous-collared sparrow *Zonotrichia capensis* (Figure 1). On a shorter-time frame social challenges, such as a territorial intruder, can cause further transient elevations in circulating testosterone levels during the breeding season (Wingfield et al., 1990; Wingfield et al., 2001).

This dynamic relationship between testosterone and behavior has been formulated into a *challenge hypothesis*, which dictates that circulating testosterone levels fluctuate according to seasonal changes in reproductive and social behaviors between breeding and nonbreeding (Wingfield, et al., 1990; Wingfield et al., 2001; Moore, 2007). It proposes that testosterone elicits aggression in defending a territory and mate guarding, which is beneficial for reproduction and there has been evidence to support this idea (Wingfield et al., 1990; Wingfield et al., 2001; Fokidis et al., 2011). Some bird species will elevate testosterone levels during inter-male competition past the concentrations required to maintain breeding physiology (DeVries et al., 2012). However, maintaining high testosterone levels can impart fitness costs to the birds, but these should not exceed the benefits of territorial aggression (Wingfield et al., 2001; Yoon et al., 2012). The specific costs of maintaining high testosterone include interference with paternal care, immunosuppression leaving the animal susceptible to already contracted diseases, and energetic costs through the rapid reduction of fat stores (Soma, 2006; Chiver et al., 2014).

Male Northern cardinals maintain relatively low plasma levels of testosterone year round with very little elevation during the breeding season (Chiver et al., 2014; Jawor, 2007). There is also evidence that the birds do not significantly elevate testosterone levels in response to a simulated territorial intrusion, however, it has been shown that male cardinals do have the physiological capacity to elevate their testosterone, particularly during aggressive encounters (DeVries et al., 2012). This was demonstrated by injection with gonadotropin-releasing hormone (GnRH), which stimulates the release of luteinizing hormone from the anterior pituitary, and in turn, stimulates testosterone production (Jawor, 2007; Deviche et al., 2010; DeVries et al., 2012; DeVries & Jawor, 2013). When breeding male cardinals were injected with GnRH, testosterone secretion increased after interaction with an aggressor (DeVries et al., 2012). Furthermore, injections of GnRH in nonbreeding male and female cardinals also resulted in increased testosterone levels (DeVries et al., 2011). As these birds maintain the physiological capacity to elevate testosterone but do not do so naturally, this suggests that other hormonal mechanisms may be responsible for territoriality in birds with year-round low testosterone. Increased testosterone also does not always coincide with increased aggression, as was demonstrated in the year-round territorial, red-throated ant-tanager *Habia fuscicauda* (Chiver et al., 2014). In this species, testosterone levels increased in the breeding season, in accordance with the challenge hypothesis, but attack rates during a simulated territorial intrusion were unrelated to increases in testosterone (Chiver et al., 2014). This suggests that though testosterone is important for mating behaviors, an alternative mechanism may be necessary to explain the aggression (Chiver et al., 2014).

### Ia. *Other Considerations for the Challenge Hypothesis*

Deviating from some of the implications of the challenge hypothesis, is the suggestion that seasonal androgen (a class of steroids that includes testosterone) responses should be evaluated separately from androgen responses resulting from intrasexual interactions between males (Goymann et al., 2007; Moore, 2007; Goymann, 2009; Fokidis et al., 2011). There is a significant difference between single-brooded (one set of offspring per breeding season) and multiple-brooded (more than one set of offspring per breeding season) bird species. In the former, male birds do not elevate plasma testosterone levels in response to a simulated intruder, while in the latter, males do show an increase despite both having seasonally elevated plasma testosterone concentrations (Goymann et al., 2007). The Northern cardinal is a multiple-brooded species (DeVries et al., 2012), so testosterone levels would be expected to increase in response to a simulated intruder. Goymann et al. (2007) proposed that the challenge hypothesis should be revised in order to encompass the essential paternal care hypothesis. This hypothesis states that organisms that require paternal care may become insensitive to androgens during the parental phase (even during male-male confrontation) because this increases reproductive success (Goymann et al., 2007; Lynn & Wingfield, 2008). A behaviorally similar species, the Northern mockingbird (*Mimus polyglottis*) is more aggressive when they are unmated or when they have older offspring present, but not during the nest-building phase when no offspring are present (Logan, 1988).

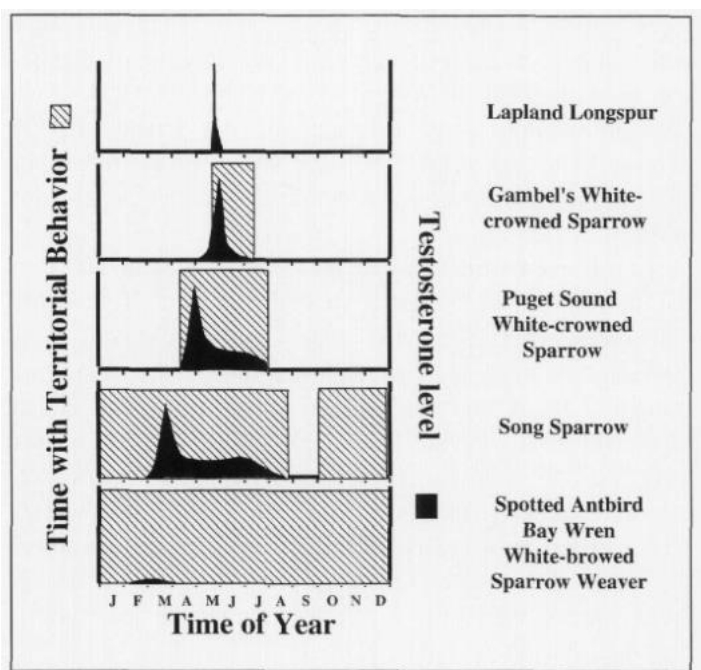
Some investigations have also focused on spatial variation in territorial aggression, such as habitat types. Several studies have shown that urban birds exhibit more territorial behavior than their rural conspecifics (Fokidis et al., 2011; Galbreath et al., 2014).



Interestingly, this behavior difference does not appear to result from changes in plasma testosterone or CORT levels (Fokidis et al., 2011). Cardinals are a common sight in the city and seem to have adapted well to urbanized landscapes. Reasons for their urban success may include an abundance of food, nesting sites, and/or more mild temperatures (Jones et al., 2010). Maintaining access to these resources may promote aggressive behavior in urban cardinals.

## II. Nonbreeding Territoriality

Territories are often established and/or maintained throughout the nonbreeding season in some species. This is thought to secure the resources that are currently present or are expected to be present once the breeding season arrives (Fokidis et al., 2011). Despite the low testosterone levels seen in the nonbreeding season, some species exhibit territorial aggression during this period (Figure 2). It has been shown in the male song sparrow, *Melospiza melodia*, which has year-round territoriality and low levels of circulating testosterone, that the brain expresses the enzymes necessary to synthesize sex steroids from steroid precursors including

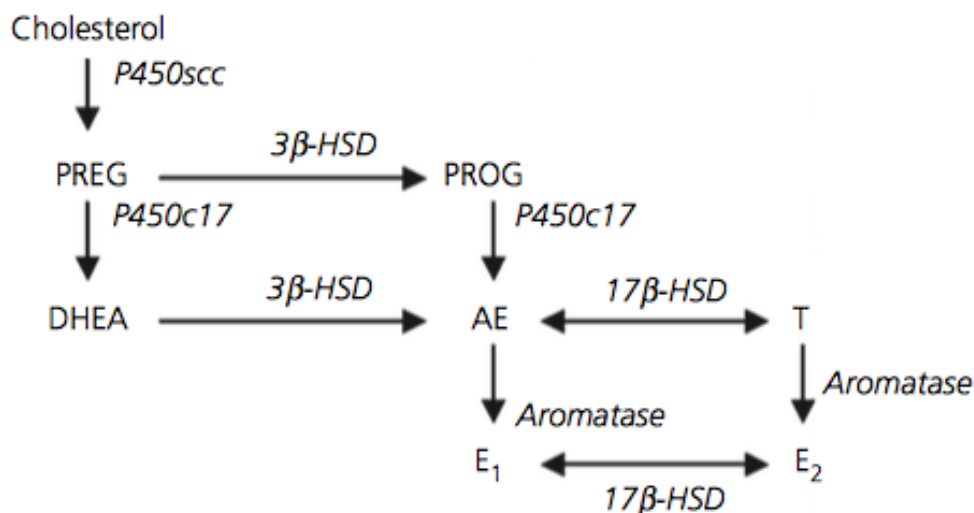


**Figure 2.** A comparison of life cycles of socially monogamous male birds from the arctic to the tropics (taken from Wingfield, Lynn, & Soma 2001). Cross hatching indicates periods of territorial behavior and black indicates relative plasma

cholesterol (Figure 3) during the nonbreeding season (Soma et al., 2000; Soma, 2006; Soma, et al., 2008; Wingfield, 2012). These enzymes include aromatase, which converts testosterone to estradiol ( $E_2$ , Figure 3) to encourage aggressive behavior in many species, including birds.

In an experiment with male song sparrows, aggressive behavior by nonbreeding birds was lessened when they were treated with fadrozole (an aromatase inhibitor), and this effect was reversed when the birds were then treated with  $E_2$ , suggesting that  $E_2$  within the brain directly regulates aggressive behavior in the nonbreeding season in these birds (Soma et al., 2000; Soma, 2006; Wacker et al., 2008). However, a study performed in a laboratory setting rather than the field found that administration of fadrozole led to an increase in aggressive behavior rather than a decrease (Wacker et al., 2008). This is likely explained by different mechanisms regulating aggressive behaviors between the lab and the field (Wacker et al., 2008). Elucidating these differences warrants further study.

The amount of energy available to the bird can ultimately influence which male will



**Figure 3.** Simplified pathway of steroid hormone synthesis (taken from Soma, 2006). Abbreviated names for steroids are located at the ends of arrows (PREG, pregnenolone; PROG, progesterone; DHEA, dehydroepiandrosterone; AE, androstenedione; T, testosterone; E1, estrone; E2, estradiol. Enzyme codes are italicized next to arrows, see Soma (2006) for details.

win during a territorial intrusion bout. Most often, the resident male bird will win against an intruding challenger (Beletsky & Orians, 1987; Beletsky & Orians, 1989). Two hypotheses that have been proposed that explain this outcome. The *value asymmetry hypothesis* states the resident wins because the territory is more valuable to them, providing more motivation to defend it (Maynard & Parker 1976). In contrast, the *resource holding potential hypothesis*, states the resident wins because they are better “fighters” than their challengers and this is independent of their motivation to defend their territory (Parker 1974). There has been some evidence to support the value asymmetry hypothesis over the resource holding potential hypothesis in Red-Winged Blackbirds, *Agelaius phoeniceus* ( Beletsky & Orians, 1987; Beletsky & Orians, 1989). Male blackbirds were withheld from their territories for variable amounts of time and when released, the factor influencing whether they regained the territory was whether the new owner was a previous neighbor or a previously non-territorial male, rather than the time they were held off the territory or the duration the new male had stayed in the territory (Beletsky & Orians, 1987). Beletsky & Orians (1987) ultimately suggested that the patterns of territory recovery demonstrated asymmetries in local knowledge and experiences between resident males and challengers, supporting the value asymmetry hypothesis. The birds that were withheld from the territory were more motivated to defend it, and so they won the challenge.

### III. Dehydroepiandrosterone

Dehydroepiandrosterone (DHEA) is a precursor to the sex steroids testosterone and estrogen (Figure 3) and has been receiving increasing attention as a potential regulator of

nonbreeding aggression (Hau, et al., 2004; Soma, 2006; Soma et al., 2008; Wacker et al., 2008). DHEA is present in mammalian blood in its largely inactive, sulfated form (DHEAS), but primarily exists in its active form in both bird circulation and within the brain (in both mammals and birds) making it more readily accessible (Webb et al., 2006). The mechanism by which DHEA acts is widely unknown, although studies on human cells and in rodents has demonstrated that DHEA can bind with very weak affinity to a variety of membrane-bound receptors (Webb et al., 2006). More likely, when circulating testosterone levels are low (such as in the nonbreeding season), the brain can metabolize circulating DHEA into active sex steroids in response to physiological indicators of season (Soma, 2006; Soma et al., 2008).

One experiment, conducted on year-round territorial spotted antbirds, *Hylophylax*

*naevioides*, in Panama involved exposing them to

simulated territorial intrusions during the

nonbreeding season (Hau et al., 2004). Here, they

reported that plasma DHEA plasma concentrations

were higher than testosterone and estradiol levels

and these concentrations increased with the

amount of time the bird was exposed to the

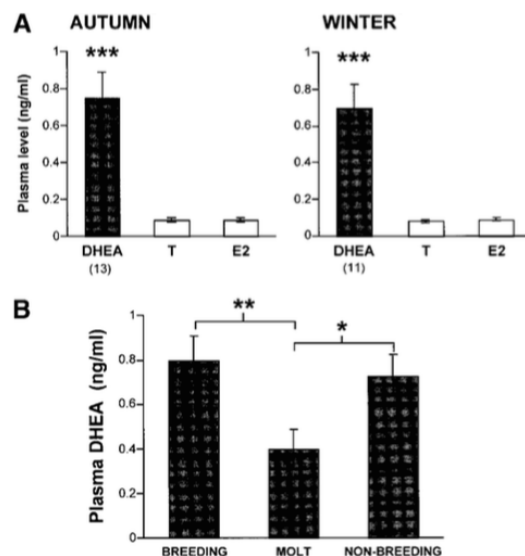
simulated intruder and their aggressive

vocalizations (Hau et al., 2004). Similarly, the male

song sparrow maintains high levels of DHEA in the

nonbreeding season, with a slight increase in the

breeding season (Figure 4), which would



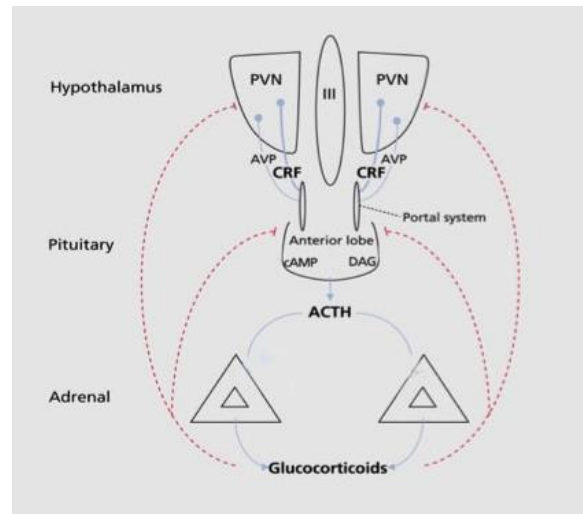
**Figure 4.** (A) Plasma dehydroepiandrosterone (DHEA) levels in wild adult male song sparrows in the nonbreeding season (autumn and winter). (B) Seasonal changes in plasma DHEA levels in wild adult male song sparrows (taken from Soma & Wingfield 2001).

correspond with its expression of year-round territoriality seen (Soma & Wingfield 2001). This combination of evidence lends support to an alternate mechanism to the regulation of aggression during the nonbreeding season involving the conversion of circulating DHEA within the brain to testosterone or estradiol. One evolutionary explanation for the increased reliance on steroid hormone precursors (i.e., DHEA) to regulate aggression is that it can elicit the beneficial territorial aggression while avoiding the energetic and immune costs of high testosterone (Hau et al., 2004).

#### IV. Corticosterone

Corticosterone is another steroid hormone (specifically a glucocorticoid) that is usually associated with stress, which is also important in the context of territoriality. Prolonged aggression against an intruder is stressful to a

bird, causing the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 5; Smith & Vale 2006). During activation of the HPA axis, the paraventricular nucleus of the hypothalamus releases corticotropin-releasing factor (CRF) and vasopressin (AVP), (Figure 5; Smith & Vale 2006). CRF then interacts with cyclic adenosine monophosphate (cAMP) in the anterior pituitary gland to release adrenocortitrophic hormone (ACTH), (Figure



**Figure 5.** Schematic representation of the HPA axis (adapted from Smith & Vale, 2006). Depicts the paraventricular nucleus (PVN), which synthesizes corticotropin releasing factor (CRF) and vasopressin (AVP). Cyclic adenosine monophosphate (cAMP) pathways release adrenocortitrophic hormone (ACTH), which stimulates release of glucocorticoids after interaction with AVP.

5; Smith & Vale 2006). ACTH then interacts with AVP to stimulate the release of glucocorticoids from the adrenal gland, (Figure 5; Smith & Vale 2006). Consequential release of CORT then helps to adjust metabolic activity and behavior in response to a changing, stressful environment (Rich, 2005; Deviche et al., 2010; Cockrem, 2013). Corticosterone and other glucocorticoids lead to decreased secretion of GnRH, which ultimately suppresses plasma testosterone levels (Deviche et al., 2010; Fokidis et al., 2011). An experiment on the European nuthatch, *Sitta europaea*, found that simulated territorial intrusions in the nonbreeding season did not elicit a testosterone response, but increased CORT (Landys et al., 2010). Like testosterone, chronic elevation of CORT can be costly through immunosuppression and the rapid depletion of energy reserves.

A common source of stress that wild animals often endure is periods of low food availability resulting in fasting or food restriction, which stimulates CORT secretion (Kitaysky et al., 2001; Herring et al., 2011). Further some studies report that CORT levels are reduced when food is readily available (Kern et al., 2005; Herring et al., 2011). As CORT usually suppresses sex steroids, if birds had steady access to food, stress could be reduced thus lowering levels of CORT and possibly increasing aggression, especially as they have the energy to fight off an intruder.

#### V. *The Northern Cardinal*

The northern cardinal is an excellent model system for the present field study examining the relationship between hormones and territorial behavior. They are very common birds that range from Central America to southern Canada (DeVries, et al., 2012; Jawor, 2007; Susan DeVries et al., 2011). The sexes are dichromatic and are easy to tell apart; males have



**Figure 6.** Northern Cardinal Plumage. The female is pictured on the left with predominately gray plumage. The male is on the right with bright red plumage. Taken from JMC Nature Photos.

bright red plumage while females are a dull, grey-brown color with only a small amount of redness in their plumage (Figure 6). Importantly, in Florida, cardinals are year-round residents that actively defend their territories, thus it is unlikely that birds will leave their territories during the duration of this experiment. Cardinals display territorial aggression year-

round due to long reproduction periods ( $6\pm$  months) that results in their monogamous behavior (Jawor, 2007; Susan DeVries et al., 2011). Long periods of territoriality are unusual for most temperate bird species, making the northern cardinal a unique exception (Jawor, 2007; DeVries et al., 2011). Displays of territoriality during the nonbreeding season can be observed in both sexes of the cardinal and these behaviors can include solitary singing, duets, bill wiping, and display flights which are easily observable and quantifiable (Jawor, 2007; Susan DeVries et al., 2011). Cardinals also maintain low testosterone levels throughout the entire year, but retain the physiological capacity to elevate concentrations higher (Chiver et al., 2014; Jawor, 2007; DeVries et al., 2012). This species is also very prone to visiting bird feeders, thus making a food supplementation study feasible. Taken together, the northern cardinal is a prime candidate for studying how food-supplementation can impact how steroids effect aggression.

## *VI. Energy & Metabolites*

This study investigates how food supplementation can alter steroid concentrations and the implications for aggressive behavior. To evaluate the energetic effects of aggression and food supplementation, circulating metabolites could be measured that indicate deposition (triglycerides) and/or usage (glycerol) of energy reserves. When a bird exerts energy, such as during aggressive behaviors, triglycerides, the primary avian fat store molecules, are metabolized and broken down into three fatty acids and a single free glycerol molecule (Berg et al., 2002). This free glycerol could then be further metabolized into glucose through gluconeogenesis within muscle and the liver (Berg et al., 2002). Thus high circulating levels of glycerol indicate a high rate of triglyceride metabolism, whereas high levels of circulating triglycerides suggest increased fat deposition into energy reserves. This study aims to test how food supplementation influences the steroid levels and aggression. By measuring higher triglyceride and lower free glycerol concentrations in blood, we can determine that birds are obtaining an energetic benefit from food supplementation.

## *VII. Experimental Design and Significance*

The goal of this study is to test how food availability and duration of aggression affect the energetic status of the Northern cardinal and to provide evidence for a DHEA-mediated mechanism of nonbreeding aggression. I will compare aggression in wild male cardinals that are either food supplemented (FS) or non-supplemented controls (CON) by observing their responses to variable durations of a song playback (ranging from two minutes to upwards of an hour) simulating a territorial intruder. This study is the first to explore how



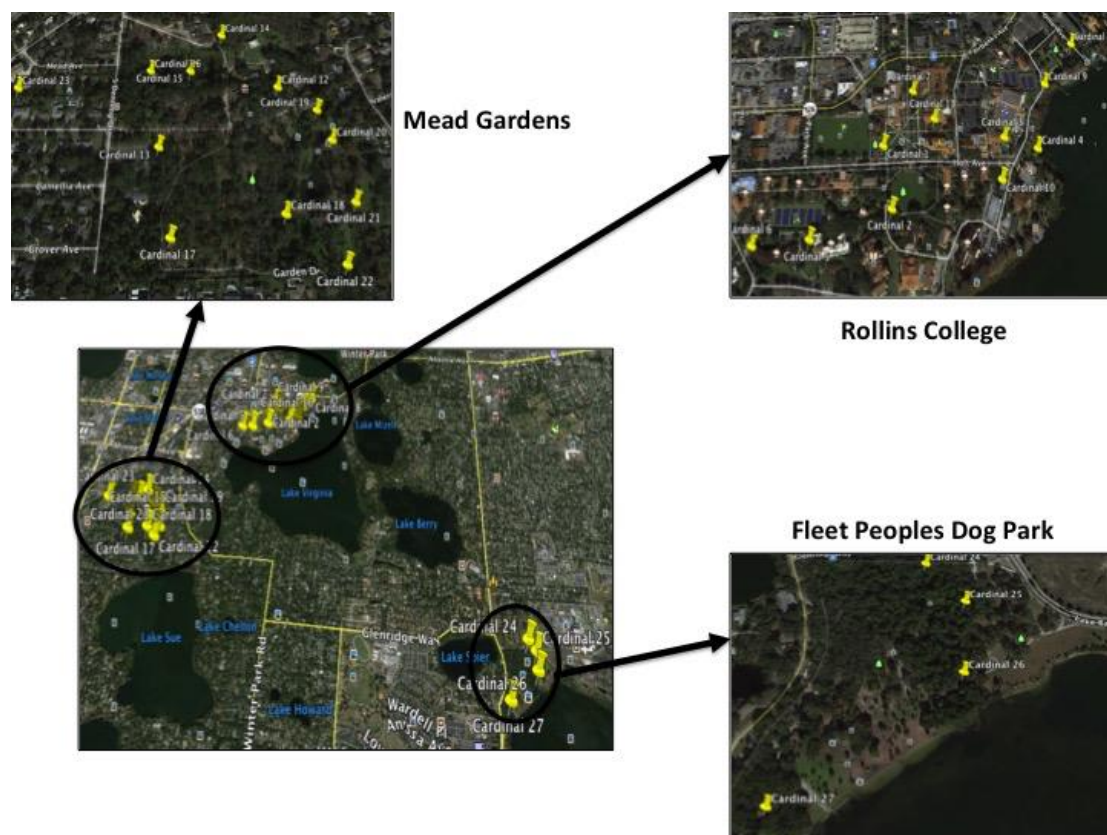
the availability of food can influence nonbreeding territorial behavior and the potential role of steroid precursors like DHEA, and stress hormones like CORT. Testosterone will not be measured, and the assumption will be made that testosterone levels will remain relatively low and not change with treatment in accordance with the existing literature on this species (Chiver et al., 2014; Jawor, 2007; DeVries et al., 2012). I hypothesize that territorial challenges will increase utilization of metabolites so that if aggression is maintained for an extended period of time, metabolite levels should favor energy usage (more glycerol and fewer triglycerides) compared to a bird who has exhibited aggression for only a short period of time. Furthermore, birds with greater food security (i.e., FS birds) will exhibit more aggression, and hence more DHEA and less CORT, while expending more energy than CON birds with less food security. Of particular interest is whether an increase in DHEA occurs with challenge, which would provide evidence for DHEA-regulated aggression in nonbreeding cardinals.

There is a surprisingly limited amount of research that uses natural systems to study aggression. This project, which is conducted in a wild bird, will contribute to this growing body of work. Furthermore, steroid precursors are gaining more attention as important “functional” molecules in multiple vertebrate species, including humans. Currently, the FDA does not regulate DHEA supplement sales for human use, but notes numerous side effects of use including increased irritability and aggressiveness (American Cancer Society, 2008). By further understanding how DHEA may regulate aggression, the sale of pharmaceutical supplements can be better regulated.

## **Materials and Methods**

### *I. Cardinal Field Survey*

This study was conducted at three field sites in Winter Park, Florida: the Rollins College campus, Mead Gardens, and Fleet Peoples Dog Park at Lake Baldwin. These sites were chosen due to proximity to our location, ease of sampling and they possessed suitable habitat for cardinals. Initial surveying began at and around sunrise from early Sept to mid-Oct 2014. Birds were located using conspecific playback recordings played (courtesy of the Cornell Lab of Ornithology's song database) over a speaker. Once a cardinal was spotted, the bird was observed for about five minutes in order to observe general aggressive responses and especially to visualize the general territory size by assessing display flights around their territory perimeter. This method minimized the likelihood of sampling the same bird twice. The approximate GPS coordinates of the center of the territory were then recorded using a GPS application for the iPhone and mapped using Google Earth (Figure 7). A total of 27 mating pairs were located using the above method.



**Figure 7.** Field sites for cardinal surveyed and captured during this study. The bottom right picture shows all three field sites in relation to each other with Rollins College the most northern site, and Fleet People's Dog Park the most southern. The top right picture is of the Rollins College Campus. Locations were mapped and pictures created using Google Earth.

## II. Food Supplementation

All cardinal pairs were assigned a unique number and a random number generator ([www.stat trek.com](http://www.stat trek.com)) was used to determine which birds would be food supplemented with wild birdseed (Pennington Seed Wild Bird Ration, Pennington Inc., Indianapolis, IN) and which birds would serve as non-supplemented controls. Ingredients for the above birdseed include white proso millet, wheat, red milo (sorghum), black oil sunflower, peanuts, and mineral oil. When two birds had overlapping territories, the method was altered so that they would either both be supplemented or both not be supplemented. In total, 14 pairs

received food and 13 pairs did not. Food supplementation involved adding four quarts of birdseed placed on a wooden board within the center of the bird's territory. Empty boards were also placed in the territories of non-supplemented birds to control for the potential effect of a novel object on a bird's territory. Food supplementation began in mid-Oct 2014 and continued for at least four weeks, (Oct through Nov) with feeders checked twice a week and replenished as necessary. Feeding continued until the adult male cardinal at the site was captured for sample collection.

### *III. Field Capture and Sample Collection*

Sample collection began in early Nov 2014, approximately four weeks after food supplementation began, and continued through the end of Dec 2014. All birds were captured between 6:30 AM and 9:30 AM. In order to capture the birds, conspecific playback recordings were used to stimulate a territorial aggressive response and lure the birds into mist nets. Often both male and female cardinals were captured using this method however this study focused on adult males. Sex was determined visually by plumage coloration. Some birds were unable to be captured due to a lack of aggressive behavior preventing them from approaching the mist net.

During conspecific playback, the time it took for the bird to respond (*hereafter* response time) and the time until the bird was captured (*hereafter* capture time) were both recorded. After capture, approximately 300  $\mu$ L of blood was collected from the right jugular vein into a heparinized 0.3 ml syringe with a 29.5 gauge needle. The birds were kept in a cloth bag and after 30 minutes, the birds were bled again using the same method to account for changes in plasma hormone and metabolite levels due to handling stress. The blood

samples were kept on ice in the field and later centrifuged to separate plasma that was stored at -80°C until assayed for steroids and metabolites.

Additional measurements were also recorded including tarsus and beak (nares to tip) lengths (to nearest 0.1 mm), the length of the wing chord (to nearest 1 mm), width of the cloacal protuberance (androgen-dependent secondary sex characteristic); pectoralis muscle and furcular fat scores (both a 5 point visual scale); and body mass (to the nearest 0.1 g). Furthermore whether the birds were molting their feathers was also recorded. Last, subjects were given a uniquely numbered US Geological Survey aluminum leg band and released at the site of capture. A total of 10 control males and 6 food-supplemented males were captured for study.

#### *IV. Free Glycerol and Triglyceride Endpoint Assay*

Measurements of free glycerol and triglycerides in plasma were made using a sequential enzymatic assay in a 96 well microplate that has been modified for use in birds (Fokidis et al., 2011). A serial dilution of a glycerol standard solution (Sigma G7793, 2.5 mg/ml triolein concentration) was used to generate a standard curve that allowed interpolation of unknown concentrations from plasma samples. The standard concentrations were as follows: 5.64 mM, 2.82 mM, 1.41 mM, 0.705 mM, 0.3525 mM, and 0.176 mM. Then 5 µl of standard or plasma sample were added to each well in duplicate. Then, 240 µL of Sigma glycerol reagent (Sigma G6428), pre-warmed to 37°C, was added to each well. The plates were incubated for 10 minutes at 37°C and then read at a primary and secondary absorbance of 540 and 750 nm, respectively. This provided the free glycerol concentration. The wells were then loaded with 60 µL of triglyceride reagent (Sigma T2449), pre-warmed

to 37°C. They were incubated again for 10 minutes at 37°C and read at the same wavelengths as above. Plasma sample concentrations were calculated from a standard curve using GraphPad v11 (GraphPad Software Inc., La Jolla, CA).

#### V. Corticosterone ELISA Assay

Plasma CORT concentration was quantified using a commercial enzyme-linked immunoassay (Detect X Corticosterone kit, K014-H1, Arbor Assays, Ann Arbor MI) according to the manufacturer's instructions. Briefly, 5 µL of plasma samples were pre-diluted 1 to 50 with assay buffer. A serial dilution of CORT stock solution was done to produce the following standard concentrations: 10000 pg/ml, 5000 pg/ml, 2,500 pg/ml, 1250 pg/ml, 625 pg/ml, 312.5 pg/ml, 156.25 pg/ml, and 78.125 pg/ml. Then, 50 µL of either standard or samples were pipetted into their respective wells. To correct samples for non-specific binding (NSB) by the CORT antibody, 75 µL of assay buffer was added *in lieu* of standard or sample. The total binding (TB) by the CORT antibody was also determined. Then 25 µL of CORT conjugate solution was added, followed by 25 µL of CORT primary antibody were added to all, except for the NSB wells. The plates were aspirated and washed four times with a wash buffer (diluted 1:20 after which 3,3',5,5'-Tetramethylbenzidine or TMB] substrate was added, followed by 50 µL of stop solution. The optical density was read at a wavelength of 450 nm. Plasma concentrations of CORT were calculated from a standard curve using GraphPad v11.

#### VI. Solid Phase Extraction

Concentrations of DHEA are considerably lower than CORT, and thus solid phase extraction (SPE) is used to purify the steroids in a sample prior to a DHEA assay. Plasma samples were first prediluted using 10 ml of deionized water. Waters C18 carbon-bonded silica cartridges (*hereafter* columns) were primed with 3 ml of 100% ethanol and subsequently equilibrated with 10 ml of deionized water. Samples were then loaded onto the columns, after which an interference elution was done using 10 ml of 40% methanol (MeOH). This interference elution removed lipids, and steroids that were glucuronated or sulphated and could potentially interrupted the findings from the ELISA assay. A final elution using 5 ml of 90% MeOH was performed to obtain the purified samples. These samples were then dried down to extracts using a speed vacuum concentrator set at 60°C for 4 hours. Dried extracts were then stored at -20°C until assayed.

#### VII. DHEA ELISA Assay

Plasma concentrations of DHEA were assessed using a commercial DHEA ELISA kit (Diametra DKO124, Immunodiagnostic Systems (IDS) Ltd, Milano, Italy). Dried extracts were reconstituted in 3 µl of absolute ethanol and 60 µl of assay buffer. A standard curve was generated using the calibrating standards provided in the kit with the concentrations: 0.5 ng/ml, 2.0 ng/ml, 5.0 ng/ml, 10.0 ng/ml, and 30.0 ng/ml. The assay was run according to manufacturer's instructions. Briefly, 25 µl of standards and samples were loaded in duplicate onto their respective wells. DHEA enzyme reagent (50 µl) was then added to all standards and samples, followed by 50 µl DHEA biotin reagent. The plates were then incubated at room temperature for 1 hour. Following incubation, the plates were washed 3 times with 350 µl of wash solution. Then TMB substrate (100 µl) was added to all wells,

and then incubated at room temperature in the dark for 20 minutes. Finally, stop solution (50  $\mu$ l) was added to all plates and absorbance was read at 450 nm. Concentrations were calculated from a standard curve using GraphPad v11.

### VIII. *Data Analyses*

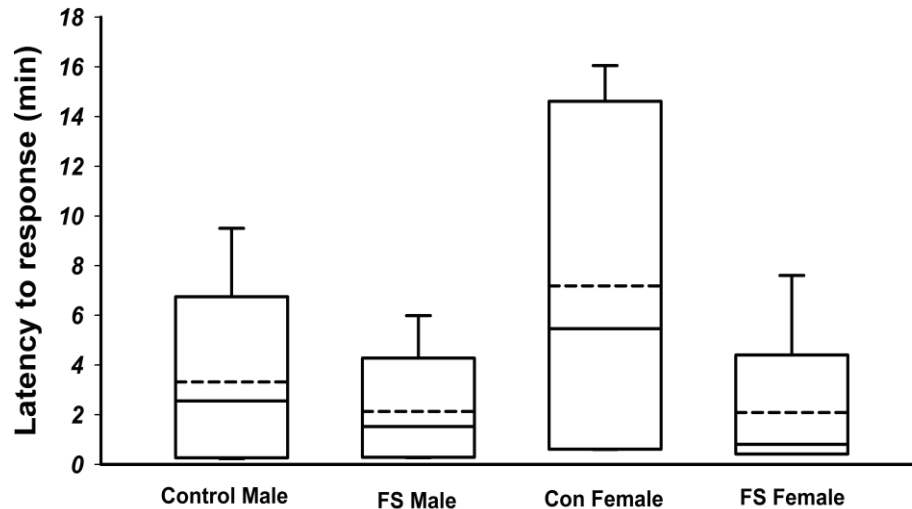
Behavioral data were compared between the sexes and treatments using two-way analysis of variance (ANOVA). Comparisons of changes in hormones and metabolites during 30 minutes of acute stress between FS and CON birds were made using repeated measures ANOVA. Other comparisons between FS and CON birds were performed using 2-sample t-tests. Data were log transformed as needed in order to pass normality tests. All statistics were analyzed with 90% confidence at an alpha value of  $P = 0.1$  and  $n = 16$  (unless otherwise stated). This more liberal alpha level takes into account the small sizes in this study. To assess how hormone levels may be influenced by energetic status, linear regressions of body mass against log-transformed values for tail, wing, tarsus, and beak lengths were performed to generate body condition indices (BCI). The standardized residuals from the regression with the strongest relationship (i.e., largest  $R^2$  value) were used in subsequent analyses relating BCI to other variables. Pearson's correlations were used to examine the relationship between hormones, metabolites and behavior. All analyses were performed using Sigma Plot 13 (Systat Inc., San Jose, CA) and all data are presented as means  $\pm$  standard error.

## **Results**

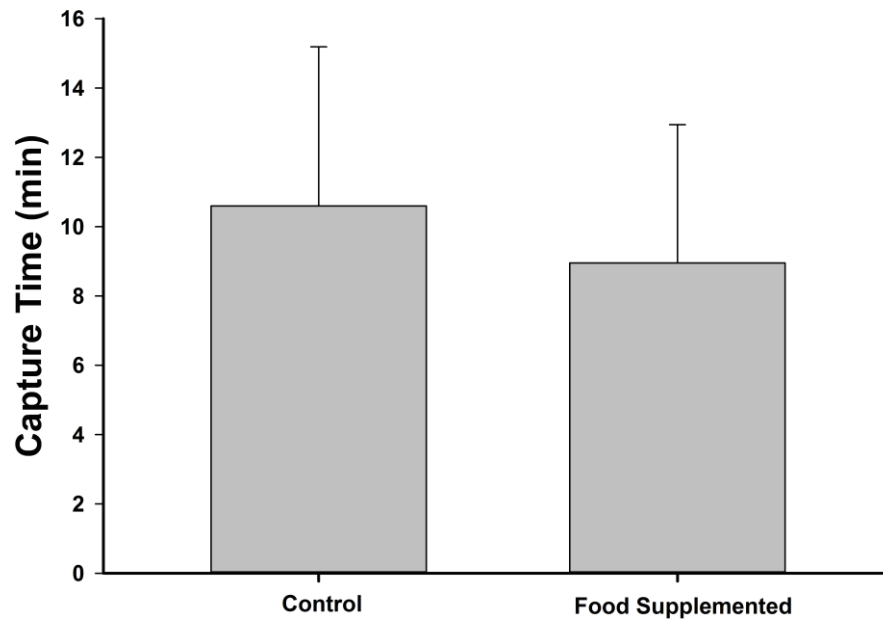
### *I. Effects of Food Supplementation on Behavior*



The latency to response (i.e., the time it took for the bird to appear after the playback for the STI began) and the time until capture were the primary behaviors measured to indicate territorial aggression. Both male and female responses were recorded. Females did not have a significantly different latency to response time than male birds (Figure 8;  $F = 0.86$ ,  $P = 0.364$ ). Further, no significant differences in latency to response were observed between FS and CON males ( $t = 0.72$ ,  $df = 14$ ,  $P = 0.242$ ) or between FS and CON females ( $t = 1.39$ ,  $df = 4$ ,  $P = 0.180$ ). Capture time, which was only recorded for males, similarly did not differ significantly between FS and CON groups (Figure 9;  $t = 0.60$ ,  $df = 14$ ,  $P = 0.278$ ). There was no correlation between latency to response to capture times ( $r = 0.24$ ,  $P = 0.372$ ). The width of the cloacal proturbance, an androgen-sensitive secondary sexual characteristic, was also not statistically different between CON and FS males ( $t = 0.06$ ,  $df = 14$ ,  $P = 0.477$ ).



**Figure 8.** Boxplot indicating the latency to response time of both food supplemented (FS) and control male and female Northern cardinals. Boxes indicate 5% and 95% percentiles, means are indicated by the solid lines, the medians are indicated by the dashed lines and error bars indicate the maximum.

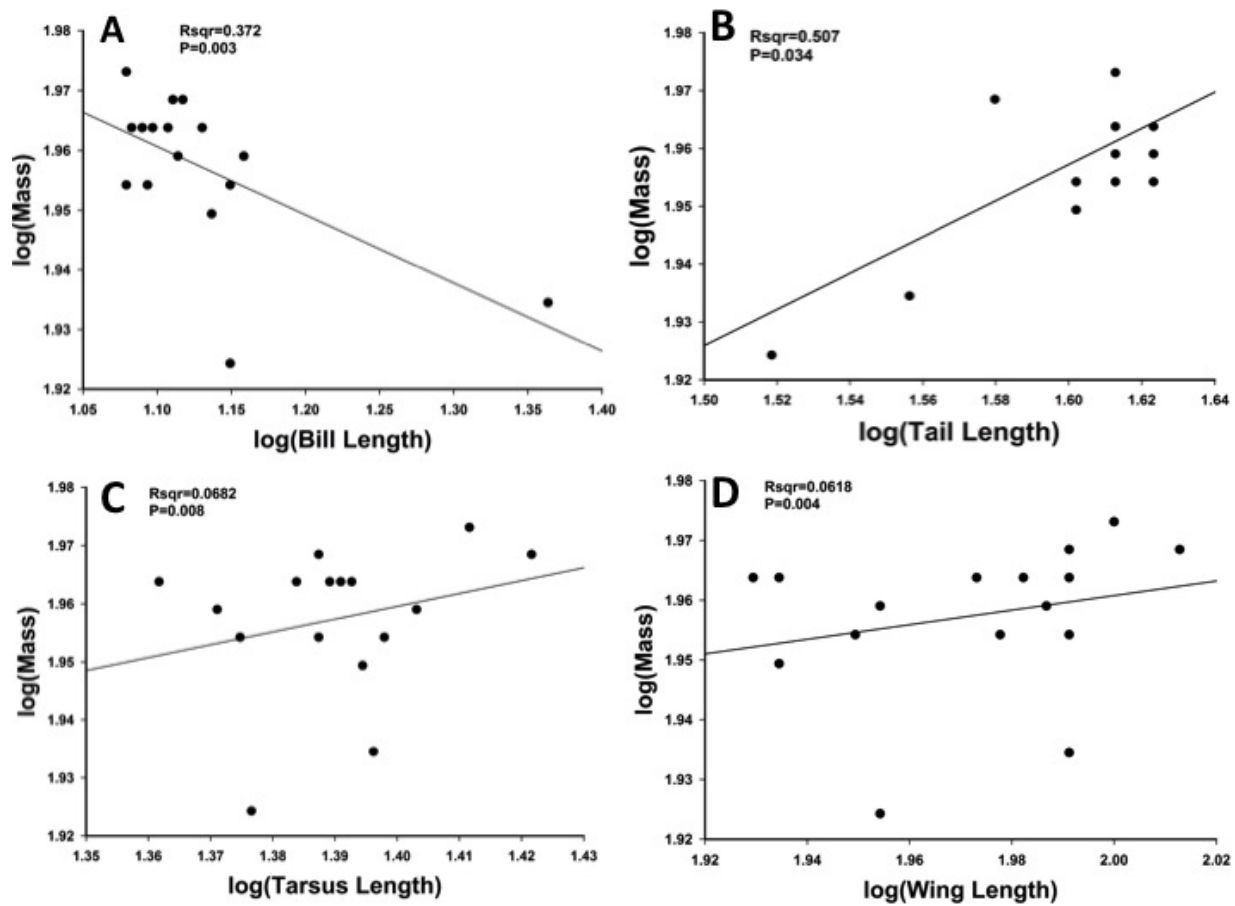


**Figure 9.** Capture time of male cardinals exposed to a simulated territorial intrusion involving playback with conspecific song.

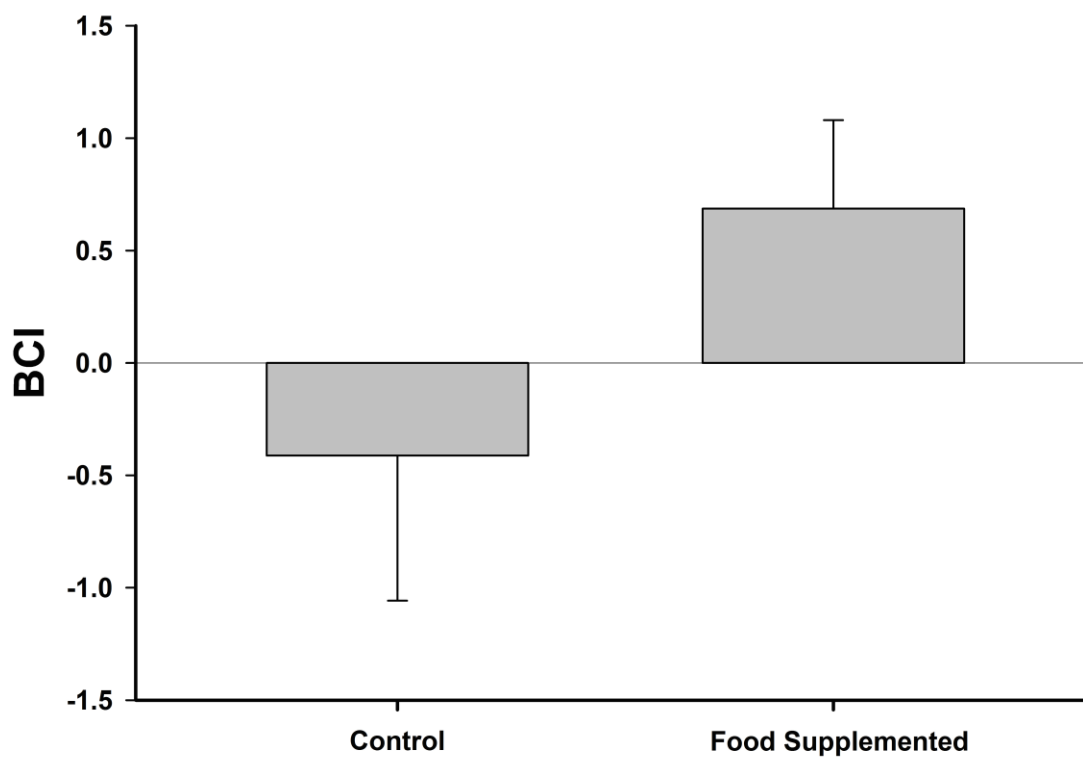
## II. Effects of Food Supplementation on Energy

All the size measurements were significantly positively associated with body mass, with the exception of beak length, which was negatively correlated (Figures 10A-D; all  $P < 0.05$ ). The regression of body mass on tail length had the strongest statistical relationship ( $R^2 = 0.51$ ,  $P = 0.034$ , Figure 10A) and thus residuals from this regression were used to generate a BCI. There was however no statistical difference in BCI between FS and CON birds (Figure 11,  $t = 0.13$ ,  $df = 14$ ,  $P = 0.896$ ). However, mass was significantly higher in FS birds compared to CON birds (Figure 12,  $t = -1.59$ ,  $df = 14$ ,  $P = 0.067$ ). Correlations between BCI and baseline concentrations for triglycerides, glycerol, and CORT did not reveal any significant trends (Figure 13A-D). However, there appears to be a non-significant trend

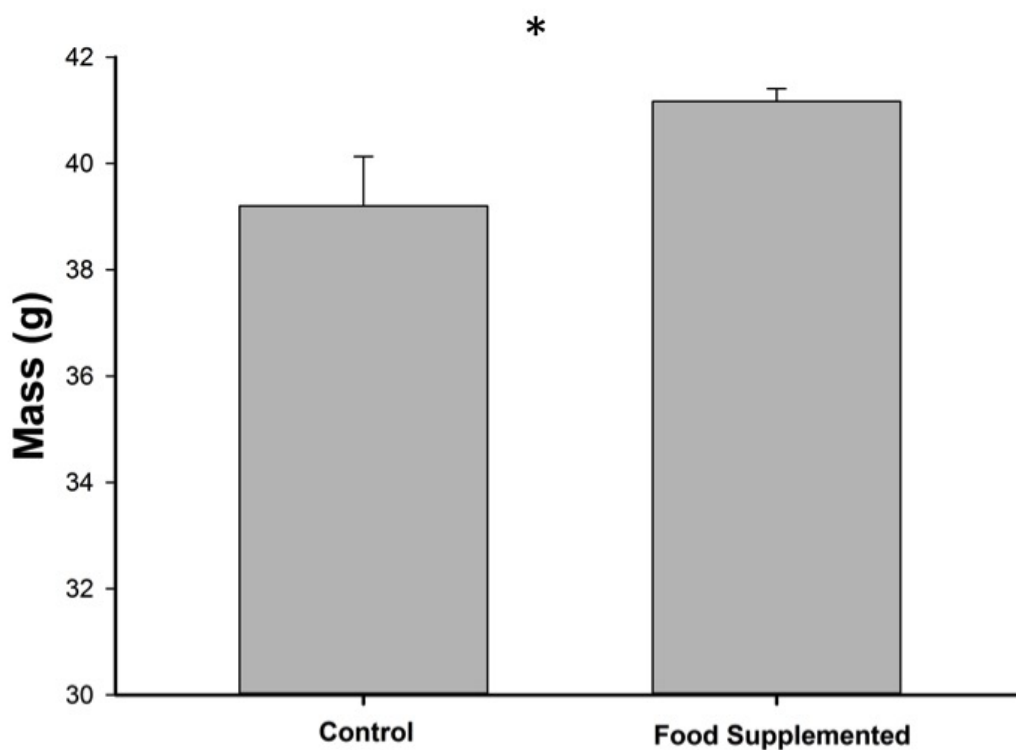
with higher baseline DHEA concentrations in birds with a better body condition (Figure 13B,  $R^2=0.16$ ,  $P=0.147$ ).



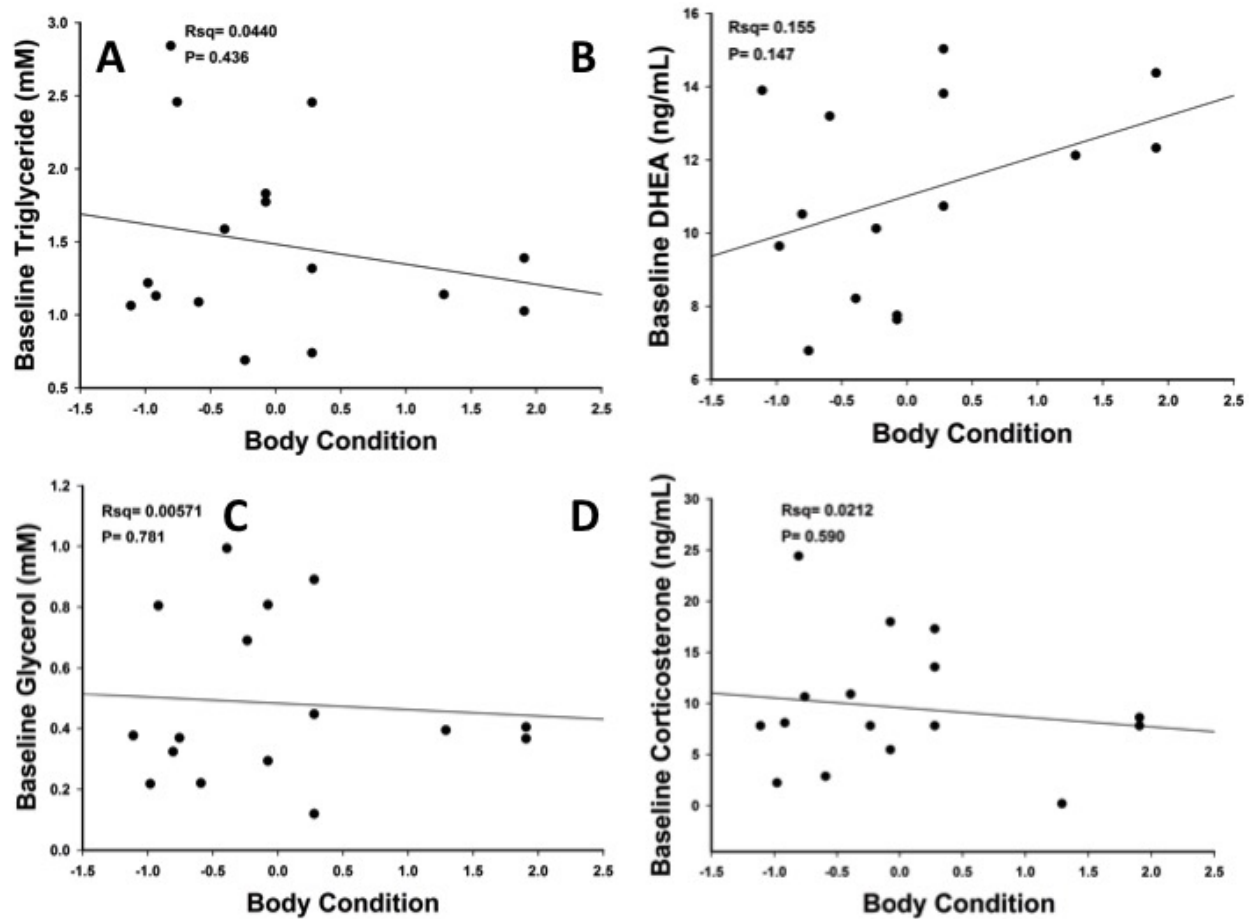
**Figure 10.** Mass plotted against (A) tail, (B) wing, (C) bill, and (D) tarsus. Data are presented as log-transformed values.



**Figure 11.** Body condition index (BCI) of male cardinals based on a linear regression of body mass on tail length.



**Figure 12.** Comparison of body mass between control and food-supplemented birds. Asterisk indicates statistical difference at  $P < 0.1$ .

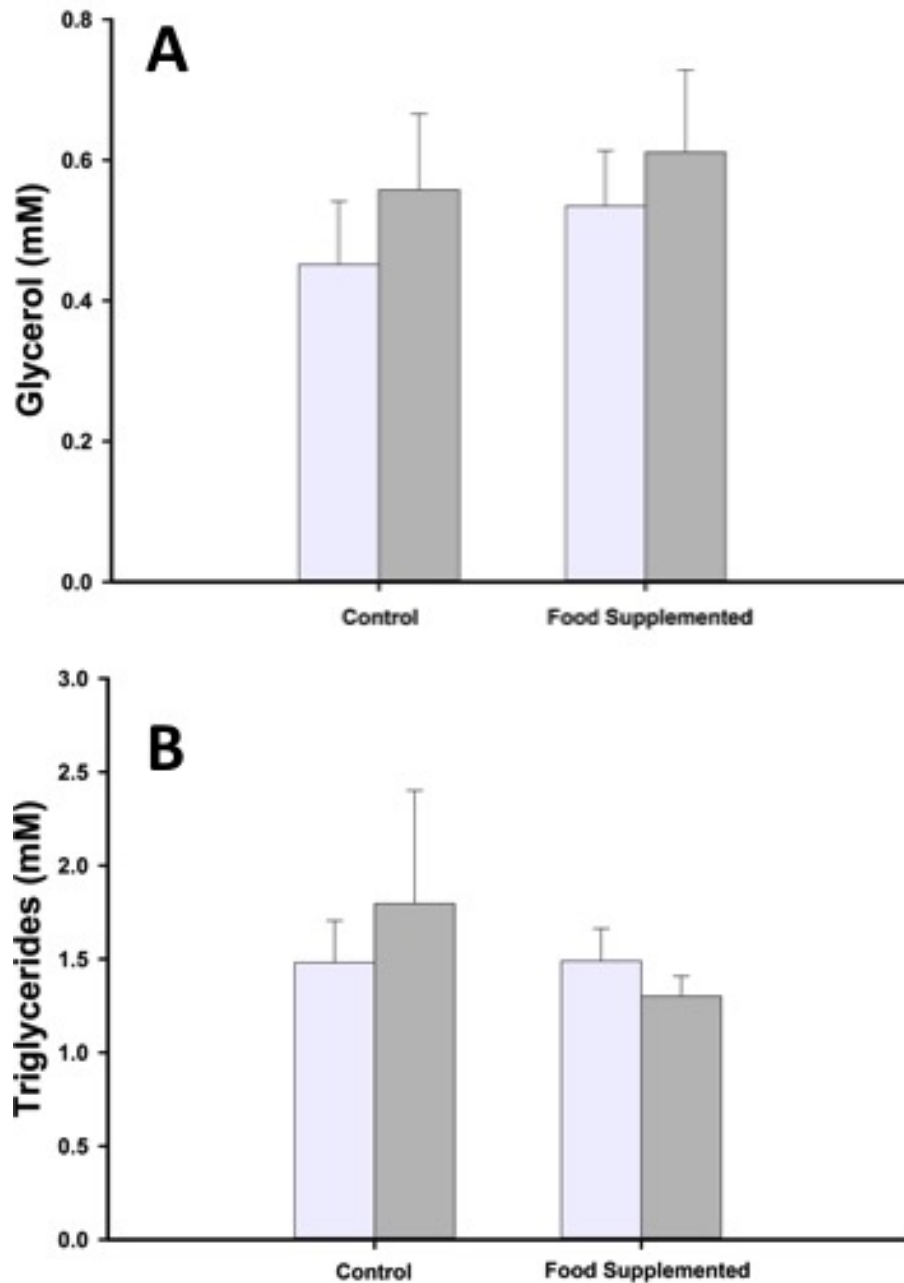


**Figure 13.** Body condition plotted against baseline (A) triglyceride, (B) DHEA, (C) glycerol, and (D) CORT concentrations.

Free glycerol concentrations appear superficially higher in FS birds compared to CON birds (Figure 14A), however this difference was not statistically significant ( $F=0.28$ ,  $P=0.604$ ). Furthermore, there was no correlation between free glycerol levels and either response ( $r=-0.153$ ,  $P=0.572$ ) or capture times ( $r=-0.218$ ,  $P=0.417$ ).

Similarly, FS birds did not differ from CON birds in their triglyceride levels ( $F=0.21$ ,  $P=0.652$ , Figure 14B). However, in CON males, triglyceride concentrations appear to have increased over the 30-minute capture period, (Figure 14B), whereas triglyceride concentrations appeared to have decreased slightly in FS males, however this was not

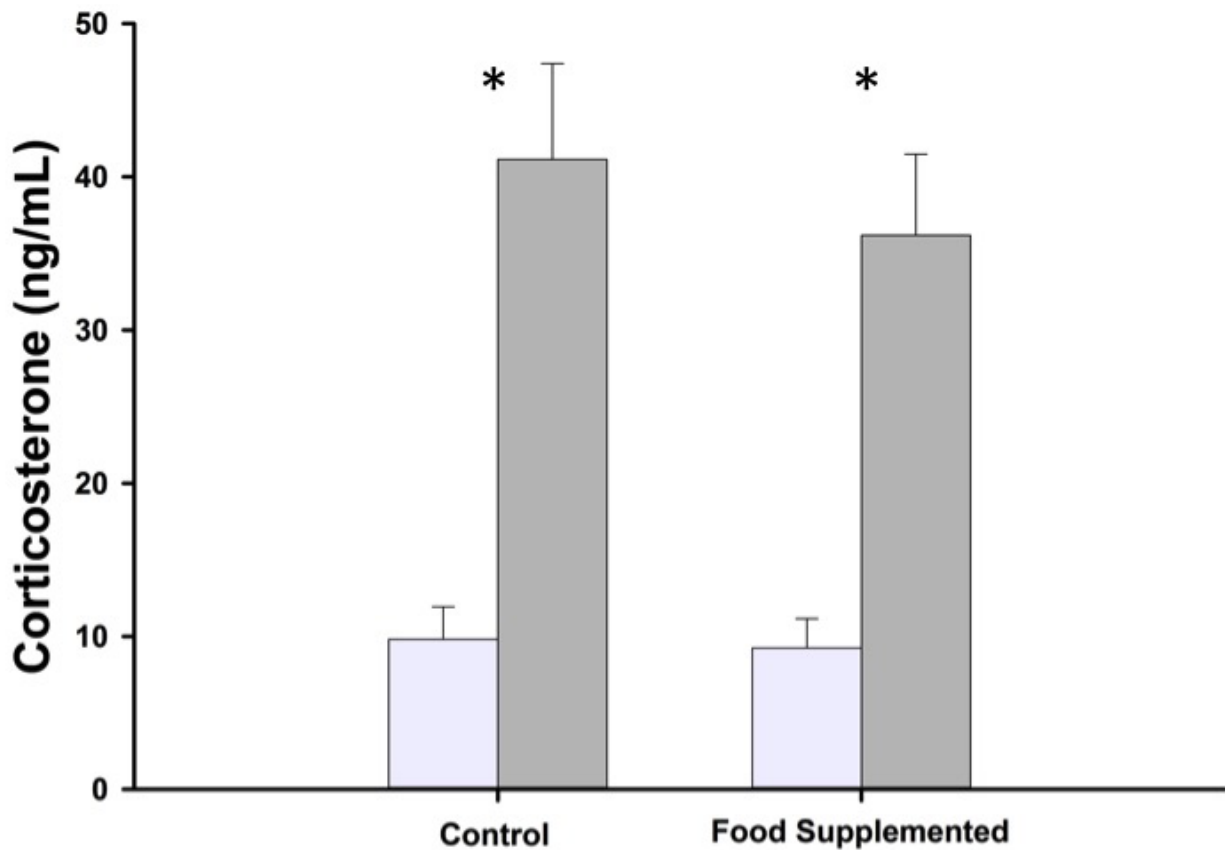
statistically significant. Again, no correlations between triglycerides and either response ( $r = -0.0927$ ,  $P = 0.733$ ) or capture times ( $r = -0.175$ ,  $P = 0.516$ ) was observed.



**Figure 14.** Average plasma glycerol (A) and triglyceride (B) concentrations in the male northern cardinal. Baseline concentrations (immediately after capture) are represented with white bars, while stressed concentrations (after 30 minutes of capture) are represented with gray bars.

#### *IV. Effects of Food Supplementation on Hormones*

Both DHEA and CORT were detectable in circulation in all the samples assayed. No significant difference in baseline CORT concentrations between FS and CON birds was observed, but after 30 minutes of capture stress, CORT levels increased to a similar degree between FS and CON birds (Figure 15;  $F = 0.48$ ,  $P = 0.498$ ). There was no relationship between CORT levels and neither plasma metabolite concentrations nor behaviors (all  $P > 0.163$ ). Pearson's correlation analysis did, however, reveal a positive correlation between baseline CORT and triglycerides ( $r = 0.55$ ,  $P = 0.028$ ).

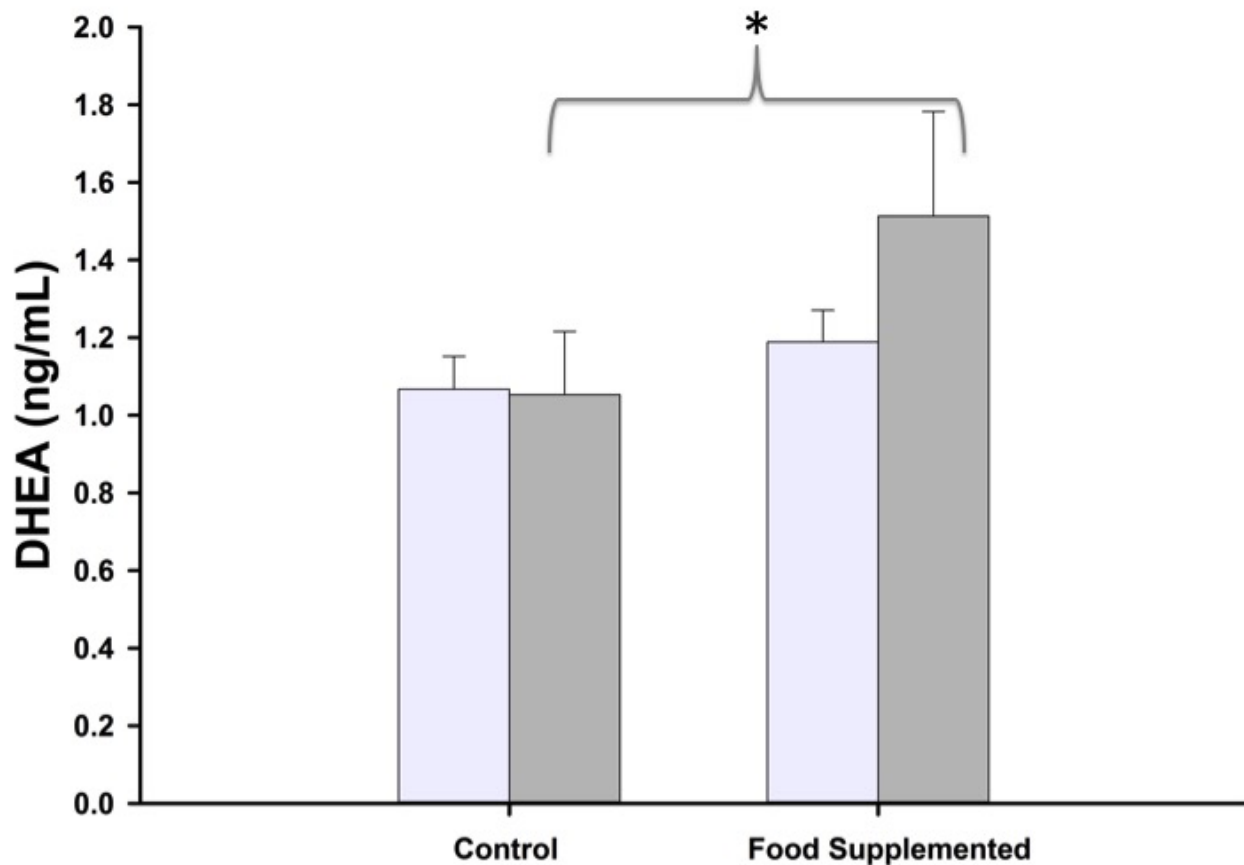


**Figure 15.** Plasma corticosterone concentration in male cardinals. Baseline concentrations (immediately after capture) are represented with white bars, while stressed concentrations (after 30 minutes of capture) are represented with grey bars. Asterisks indicate significant differences.

DHEA concentrations did not increase with handling stress in CON birds ( $n = 15$ ,  $F = 1.72$ ,  $P = 0.211$ ), but trended towards higher stress DHEA concentrations in FS than CON

males (Figure 16), however, this difference was not significant ( $t=1.685$ ,  $df= 8$ ,  $P= 0.106$ ).

DHEA concentrations were not correlated to either CORT concentrations nor aggressive behaviors ( $P > 0.264$ ). However, DHEA concentrations were significantly negatively correlated to triglyceride concentrations ( $n=16$ ,  $r = -0.56$ ,  $P= 0.024$ ).



**Figure 16.** Plasma DHEA concentration in male cardinals. Baseline concentrations (immediately after capture) are represented with white bars, while stressed concentrations (after 30 minutes of capture) are represented with grey bars. Asterisk indicates a statistical difference.

## Discussion

Territoriality during the nonbreeding season is a behavior observed across many bird species, and may function secure scarce yet necessary resources, such as food (Fokidis et al., 2011). Extensive research has been done to understand the endocrine mechanisms



regulating territoriality during the breeding season, which serves to secure a partner but, there is far less research nonbreeding territoriality. The Northern cardinal exhibits nonbreeding territorial aggression that is not attributable its year-round low testosterone profile (Jawor 2007). This study attempted to explain the regulation of nonbreeding aggression via a DHEA-mediated mechanism that is known to be influenced by food availability, and tested using a food supplementation experiment. Both FS and CON birds exhibited clear aggressive responses to song playback, but although graphically a tendency for faster response times in food supplemented birds in both sexes is apparent, these differences were not statistically significant. As expected there was also not a significant difference in the width of the cloacal proturbance in FS and CON males. This is important in that it both confirms that all birds were nonbreeding, but as it is an androgen-sensitive reproductive structure it also suggests testosterone levels were low and likely undetectable in circulation (Soma, 2006), thus ruling out testosterone as a mediator of the nonbreeding aggression observed in response to playback. Consistent with this, several of the study males in the presence of the female, were observed securing the feeders immediately upon exposure to STI as opposed to following their female mate, suggesting they were engaged in defending their resources within the territory, rather than their partner specifically. Singing was seldom observed in either group, likely because this is a behavior that is normally used during the breeding season to attract partners. Frequency of other behaviors, such as display flights and bill wiping, were not recorded as previous studies have shown that the latency to response to playback is a good predictor of aggressiveness (Fokidis et al. 2011), but perhaps future studies may want to keep track of these behaviors.

Food supplementation did not appear to affect circulating triglyceride nor glycerol levels, which contradicts our prediction that the former would be higher in FS birds compared to CON birds and vice versa for the latter. Furthermore, these levels did not significantly change in response to handling stress however there was a tendency towards an increase in glycerol levels with stress, which could suggest a mobilization of energy stores. There were also no clear associations between metabolites and the duration of the STI. Metabolites can be influenced by immediate diet and as birds were captured in the morning, the metabolite profile may reflect recent dietary intake rather than an overall indication of energetic status (Guglielmo *et al.*, 2004). Since food supplemented birds were heavier, on average, than control birds, it is likely that food-supplementation did effectively improve the energetic status of the bird.

As expected, there was a CORT stress response to capture and handling for both CON and FS birds, however the magnitude of the stress response was the same in both groups. Other studies report that food supplementation lowers baseline CORT levels (Kitaysky *et al.*, 2001; Herring *et al.*, 2011) and can alter the stress response (Rich, 2005; Deviche *et al.*, 2010; Cockrem, 2013 ). The lack of a difference in this study may have several explanations. One explanation is that although food security eliminates the stress of finding resources, an intruder on the territory may still act as a stressor causing a change in CORT levels that remove any effect of the supplementation itself. However, in this study we did not observe any relationships between CORT and the time taken to capture the bird, suggesting that responding to the STI alone will not change CORT profiles. Another explanation is that if resources were not scarce within the territories of CON birds, then we could not expect them to exhibit different CORT concentrations than FS birds. As all three

field locations were in relatively urban environments, all territories may have been already “saturated” with food, regardless of the treatment group, and so food insecurity would not be a source of stress for the birds. If this study could be replicated in a rural environment, results between groups may be more significant.

Interestingly, DHEA fluctuations appeared to be independent of CORT concentration, demonstrating that CORT does not inhibit DHEA production as it does with testosterone (Deviche et al., 2010; Fokidis et al., 2011). DHEA remained relatively stable during acute stress in control birds, with only a slight decrease after thirty minutes of capture. Conversely, food-supplemented birds had higher concentrations of DHEA after the acute stress of capture and handling, although this was not statistically significant. In song sparrows, DHEA sampled from the jugular vein (same as done in this study) decreased with acute stress in breeding birds, but increased during molt (Newman and Soma 2008; Newman et al. 2009). The increase in DHEA with stress may correspond with the greater apparent aggression observed in FS cardinals. Together these data suggest DHEA in circulation can vary in accordance with acute stress, but this may be dependent upon context or life-history stage. During capture, FS birds appeared to have continued synthesizing and secreting DHEA, which may stem from having a higher body condition, thus decreasing the energetic cost of steroid synthesis. Beyond its proposed role in aggressive behavior, another function of DHEA during stress may be to play a neuroprotective role preventing excessive damage by glucocorticoids, such as corticosterone (Newman and Soma 2008). While measuring testosterone on the samples of the present study may have been ideal, previous research would have suggested the levels would be undetectable. Thus it still plausible that DHEA may be the hormone responsible

for nonbreeding aggression. However, it is advisable that future studies should include profiles of both hormones if enough plasma can be collected, which was not the case in this study.

The negative relationship between stressed DHEA concentrations and baseline triglyceride concentrations suggests that as aggression increases, there is a decrease in triglyceride concentrations (i.e. increased usage of energy). This supports part of the proposed hypothesis, in that sustained aggression results in lower triglycerides, because maintaining the behavior is energetically costly for the bird. Unfortunately, there was not a significant correlation with glycerol concentrations, which would have further solidified support of the hypothesis.

The majority of these results did not show significant difference even when evaluated at 90% confidence rather than 95%, which can likely be attributed to a small sample size ( $n = 10$  and  $n = 6$  for CON and FS birds, respectively), combined with a large amount of individual variation. Future studies should further explore the relationship between DHEA, food availability and aggression, perhaps by conducting a similar study in a rural environment. Nonetheless, the results of this study provide interesting implications for the nature of nonbreeding territoriality. First, there is some support from this thesis for the resource holding potential hypothesis (Parker 1974) over the value asymmetry hypothesis (Maynard & Parker 1976). The birds that were FS in this study were heavier, and were perhaps able to respond to an intruder faster (i.e., more aggressively) than CON birds that were not food-supplemented. This would, in effect, make the food-supplemented birds better “fighters,” as proposed by the resource holding potential hypothesis. However, the goal of this study was not to directly test either of these hypothesis and so no concrete

conclusions can be drawn regarding the two hypotheses, particularly in light of the limited statistical power of our study. Further work can explore the likelihood that a FS bird will regain its territory compared to a CON bird, and whether this may differ between species(e.g., red-winged blackbirds and cardinals).

This study may also provide sufficient evidence to warrant modification of the challenge hypothesis. It has already been proposed that seasonal hormonal fluctuations should be evaluated separately from fluctuations due to intrasexual competition (Goymann et al., 2007; Moore, 2007; Goymann, 2009; Fokidis et al., 2011) and this study further supports this idea. The measurements taken during the course of this study were the result of intrasexual competition during the nonbreeding season and it is apparent that increased aggression does not always correspond with an increase in testosterone, which refutes a predominant assumption of the challenge hypothesis (Wingfield, et al., 1990; Wingfield et al., 2001; Moore, 2007). In time, the challenge hypothesis may perhaps be extended to include the roles of other steroids including DHEA.

This study was largely unsuccessful, although suggestive, in establishing a link between food security and aggression through a DHEA-regulated mechanism due to statistical discrepancies. However, it provided some evidence that this link may exist and could be further demonstrated with more work utilizing larger sample sizes and possibly longer periods of food-supplementation. This study was limited in time available and the time of year that it was conducted, so it was not feasible to collect a large number of samples or food supplement for longer than four weeks. However, the need for cardinals to secure resources, especially food, within their territory is important and is a driving force of aggression during the nonbreeding season. This is likely why some species establish

territories before the breeding season or maintain them year-round to insure that they will have a steady access to food during the breeding season and have a greater energetic status than competitors once the breeding season begins.

DHEA-regulated aggression in birds can be applied to non-avian species as well, and has received increasing attention for its role in mammals, even humans, although DHEA predominately exists in its inactive, sulfated form in mammals (Webb et al., 2006). As a precursor to the predominant steroid hormones, testosterone and estrogen, DHEA is actually the most abundant steroid found within mammals (Shealy, 1995). Multiple studies have shown that there is a decline in DHEA with age in people, often known as “adrenopause”, and similar declines are seen in individuals who have diseases such as cancer and Alzheimer’s disease (Shealy 1995). DHEA may protect tissues from excessive glucocorticoid damage, and may also enhance the immune system (Shealy, 1995). This is the primary drive behind the sale of DHEA as an unregulated supplement, and yet much remains unknown concerning the physiological processes that DHEA is involved in.

The goal of this study was to determine whether energetic status influenced nonbreeding territoriality in a model organism, the northern Cardinal through an effect of DHEA. However, limited statistical power in this study resulted in no significant differences in metabolites, CORT, or DHEA concentrations being observed between FS birds (high energetic status) and CON birds (lower energetic status). Nonetheless, this thesis provides trends that demonstrate that DHEA is a dynamic steroid precursor that can vary in circulation in response to environmental conditions and in particular to stress. When examining trends alone, barring statistical analysis, FS increased both body mass and the aggressive response to an STI, while also increasing DHEA secretion during stress. These

were all predictions made in the study thus suggesting DHEA may be involved to some degree in the regulation of nonbreeding territoriality in Northern cardinals.

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