SONG AS AN HONEST INDICATOR OF DEVELOPMENTAL STRESS IN SONG SPARROWS

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by

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Abstract

The Developmental Stress Hypothesis proposes that the honesty of birdsong is maintained by costs incurred during development, such that song in adulthood reflects exposure to early-life stressors. The purpose of this thesis was to provide a rigorous test of the Developmental Stress Hypothesis in song sparrows (Melospiza melodia). My three objectives were to determine the long-term effects of early-life stress on: 1) physiological traits (Chapters 2, 3, and 4); 2) male song production (Chapter 5); and 3) the response of females to song (Chapter 6). Nestlings were hand-reared in captivity under one of three treatment conditions: control, food restriction, or treatment with corticosterone (CORT). Exposure to both stressors affected nestling growth, standard metabolic rates, immune function, and endocrine regulation. There were pronounced sex differences in the effects of early-life stress on physiological traits. For example, females exposed to either stressor had lower plasma estradiol levels, but CORT-treated males had higher basal testosterone levels, showing that early-life stress has opposite effects on plasma sex steroid levels in males and females. Exposure to early-life stress affected male song production. Males exposed to either stressor sang less complex song and food-restricted males sang less accurate copies of the tutor song. Neither stressor affected song stereotypy or trill performance, however, suggesting that developmental stress may not affect vocal performance in this species. Females exposed to either stressor were less selective in their behavioural response to conspecific versus heterospecific song and to high versus low complexity song. This was paralleled by differences in levels of the immediate early gene Zenk in auditory forebrain regions. Finally, male song production was significantly related to some physiological measures. Males with a stronger swelling response to phytohemagglutinin sang more complex song and produced
higher quality trills. These results provide support for the Developmental Stress Hypothesis and add to a growing body of evidence showing that early-life stress programs many physiological and neural systems.

**Keywords**

corticosterone, developmental programming, Developmental Stress Hypothesis, food restriction, glucocorticoid, nutritional stress, songbird, song sparrow, stress
Co-Authorship Statement

Chapter 1 of this thesis was written by myself and is not published.

A version of Chapter 2 has been published: Schmidt KL, MacDougall-Shackleton EA, MacDougall-Shackleton SA, 2012. Developmental stress has sex-specific effects on nestling growth and adult metabolic rates but no effect on adult body size or body composition in song sparrows. *J Exp Biol*, 215, 3207-3217. Elizabeth and Scott MacDougall-Shackleton helped collect data and edited the manuscript.

Chapter 3 of this thesis will be submitted for publication. Beth and Scott MacDougall-Shackleton and Shawn Kubli will be coauthors as they helped collect data and perform assays to assess immune function.

A version of Chapter 4 is currently in review: Schmidt KL, MacDougall-Shackleton EA, Soma KK, MacDougall-Shackleton SA. Developmental programming of the HPA and HPG axes by early-life stress in male and female song sparrows. *Gen Comp Endocrinol*, in review. Beth and Scott MacDougall-Shackleton and Kiran Soma are coauthors on this paper as they helped collect data, perform hormone assays, and edited the manuscript.

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Chapter 7 of this thesis was written by myself. I also performed all data analyses. This chapter is not published.

Chapter 8 of this thesis was written by myself and is not published.
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List of Abbreviations

AIC: Akaike information criterion
ACTH: adrenocorticotropic hormone
ATCC: American Type Culture Collection
CFU: colony-forming unit
CMM: caudomedial mesopallium
CORT: corticosterone
CRH: corticotropin-releasing hormone
d: day of age
DLM: the dorsolateral nucleus of the anterior thalamus
dNCM: dorsal caudomedial nidopallium
FA: fluctuating asymmetry
GnRH: gonadotropin-releasing hormone
H:L: heterophil:lymphocyte
HL-HA: hemolysis-hemagglutination
HPA: hypothalamic-pituitary-adrenal
HPG: hypothalamic-pituitary-gonadal
LH: luteinizing hormone
LMAN: the lateral magnocellular nucleus of the anterior nidopallium
LSD: least significant difference
NCM: caudomedial nidopallium
nXIIIts: the tracheosyringeal portion of the hypoglossal nucleus
PBS: phosphate buffered saline
PCA: principal component analysis
PHA: phytohemagglutinin
PMR: peak metabolic rate
QMR: quantitative magnetic resonance
RA: robust nucleus of the arcopallium
REML: restricted maximum likelihood
SMR: standard metabolic rate
SPCC: spectrogram cross-correlation
TSB: tryptic soy broth
vNCM: ventral caudomedial nidopallium
Zenk: zif268, EGR-1, NGFI-A, krox24
Chapter 1

1. Introduction and Literature Review

The main objectives of this thesis were to determine the long-term effects of early-life stress on a variety of physiological traits in song sparrows, several features of male song production, and the response of female song sparrows to song. However, first I will provide a brief review of song learning, the neural control of birdsong, the function of birdsong, the evolution of female song preferences, and the Developmental Stress Hypothesis.

1.1. Song learning

Most species of songbirds (suborder Passeri, also called oscine birds) learn their species-specific song early in life from adult tutors (Catchpole and Slater, 1995). Vocal learning is rare among animals and in addition to songbirds only occurs in humans, cetaceans, elephants, some bats, and two other orders of birds (parrots and hummingbirds; Jarvis, 2006). The first experimental evidence that songbirds learn their song came from studies showing that males that are deafened early in life or raised in isolation develop highly atypical songs (Marler, 1981; Marler and Peters, 1977; Thorpe, 1958). In addition, in many species, birds develop songs that closely resemble the tutor songs they were exposed to during development (Marler and Peters, 1987; Nordby et al., 2000). However, not all species of songbirds require exposure to song early in life in order to produce adequate song in adulthood. For example, both grey catbirds (Dumetella
carolinensis [Kroodsma et al., 1997]) and sedge warblers (Acrocephalus schoenobaenus [Leitner et al., 2002]) appear to develop normal song repertoires when reared in song-isolation conditions.

For many species of songbirds that do learn their song, exposure to the song tutor has to take place early in life during a sensitive period in development for adequate learning to occur (Bohner, 1990; Marler and Peters, 1987). The length and timing of the sensitive period for song acquisition varies considerably amongst species (Beecher and Brenowitz, 2005). In addition, some species do not learn new songs beyond their first year and once their song repertoire has crystallized it remains fixed throughout adulthood. These species are called closed-ended or age-limited song learners (e.g. zebra finch [Taeniopygia guttata], white-crowned sparrow [Zonotrichia leuкоphrys]). Other species, referred to as open-ended learners (e.g. European starling [Sturnus vulgaris], canary [Serinus canaria domestica]), continue to learn songs throughout life (Beecher and Brenowitz, 2005; Catchpole and Slater, 1995).

Song learning can be broken down into two stages (Fig 1-1). During the first stage, referred to as the sensory or memorization phase, the young bird forms memories of its tutor’s songs (Marler, 1997). During the second phase, referred to as the sensorimotor phase, the juvenile bird begins producing highly variable imitations of the tutor’s songs referred to as subsong in an attempt to match their vocal output to the stored auditory memories (Marler, 1997). These early imitations gradually develop into stereotyped or “crystallized” versions of the species-specific song. The length and timing of these two stages varies and can be separated in time for some species and overlap in others. The timing of the song-learning period has been well studied for some species
including zebra finches (Eales, 1985), white-crowned sparrows (Marler, 1970), swamp sparrows (*Melospiza georgiana* [Marler and Peters, 1988]) and song sparrows (*Melospiza melodia*). In song sparrows, the sensory phase begins as early as 20 days post-hatch (d20) and is mostly complete by d90 (Marler and Peters, 1987). However, the sensory phase can be considerably lengthened in song sparrows if young birds are tutored with live singing males, as opposed to recorded songs, and may extend upwards of d300 (Nordby et al., 2001; Nordby et al., 2000). Song sparrows begin producing subsong around d50

**Figure 1-1** Overview of the stages of song learning in song sparrows. The boxes indicate timing and the approximate duration of the sensory and sensorimotor stages, as well as song crystallization. Song sparrows are close-ended song learners and do not learn new songs past one year of age. The dotted arrow next to the sensory phase is to indicate that this stage can be lengthened if young song sparrows continue to be exposed to adult singing males throughout their first fall and winter. This could occur for laboratory-raised birds exposed to live tutors or free-living non-migratory populations where adults sing all year round. d = day of age.
(personal observation) and their repertories are crystallized by their first breeding season and remain fixed in adulthood. In support of this, Norby et al. (2002) recorded 24 free-living adult male song sparrows in 2-4 consecutive breeding seasons and males did not alter their song repertoires between years after it had been crystallized in their first breeding season.

### 1.2 Neural control of song

The learning and production of song are controlled by a discrete series of interconnected brain nuclei referred to as the song-control system (Zeigler and Marler, 2008). The song-control system consists of two main pathways (Fig 1-2): the motor pathway and the anterior forebrain pathway. In the motor pathway, the nucleus HVC sends projections to the robust nucleus of the arcopallium (RA). RA then innervates a motor nucleus (the tracheosyringeal portion of the hypoglossal nucleus; nXIIIts), which then projects to the syrinx, the avian vocal organ. This pathway is critical for the production of song. For example, lesions to HVC or RA disrupt adult song production in canaries (Nottebohm et al., 1976) and zebra finches (Simpson and Vicario, 1990). The anterior forebrain pathway begins with HVC, which sends projections to area X. Area X innervates the dorsolateral nucleus of the anterior thalamus (DLM), which sends axons to the lateral magnocellular nucleus of the anterior nidopallium (LMAN). Finally, LMAN innervates RA. The anterior forebrain pathway is important for song learning (Scharff and Nottebohm, 1991). For example, lesioning area X in juvenile zebra finches disrupted song learning and caused juvenile birds to produce vocalizations characteristic of
younger birds. (Sohrabji et al., 1990).

Figure 1-2 Simplified schematic of the song-control system showing the motor pathway (dotted arrows) and the anterior forebrain pathway (solid arrows). RA = robust nucleus of the arcopallium, DLM = the dorsolateral nucleus of the anterior thalamus, LMAN = the lateral magnocellular nucleus of the anterior nidopallium, nXIIIts = the tracheosyringeal portion of the hypoglossal nucleus.

The importance of the song-control system to song learning is further supported by the fact that development of the song-control system shows considerable overlap with the timing of song acquisition (Bottjer et al., 1985). For example, in zebra finches, the sensory phase for song learning lasts from about d20-d65 and the motor phase begins around d30 with song becoming fully crystallized by d90 (Immelmann, 1969; Slater et
al., 1988). The volumes of several song-control nuclei, including HVC, RA, and area X, increase substantially between d10 and d60 (Bottjer et al., 1985; Bottjer et al., 1986). In addition, many of the connections between the different nuclei also develop during the period of song acquisition. For example, RA begins receiving functional projections from HVC on d25 in zebra finches (Mooney and Rao, 1994). Very little is known about the timing of development of the song-control system in other species of songbirds. However, in many species, song does not become crystallized until much later than it does in zebra finches so it seems likely that development of the song-control system continues until at least d65, if not longer.

Adequate development of the song control-system may be important because variation in the size of the song-control nuclei in adulthood may be related to variation in the quality of song production. For example, the volume of HVC may be positively correlated with song complexity between species (Devoogd et al., 1993) and the volume of both HVC and RA may be positively correlated with song complexity within species (Garamszegi and Eens, 2004). However, this relationship may not hold true for all species. For example the size of HVC was not related to song complexity in red-winged blackbirds (*Agelaius phoeniceus* [Kirn et al., 1989]). In addition to song complexity, the volume of the song-control nuclei may be related to other song features such as the length of song phrases (Airey and DeVoogd, 2000) or song bouts (Bernard et al., 1996) and the stereotypy of note structure (Smith et al., 1997). Furthermore, neural attributes other than the volume of the song-control nuclei may be related to song features, including the number or density of neurons in the nuclei as well as the size of neurons (Smith et al., 1997).
1.3 The function of birdsong

In most temperate-breeding species of songbirds that have been studied only males sing, or, if females do sing they sing less than males. Males sing for two main purposes: to defend territories and to attract mates (Catchpole and Slater, 1995). The role of song in territory defense was illustrated by a study of Scott’s seaside sparrows (Ammodramus maritimus peninsulae) in which males that were temporarily muted were more like to lose their territories (McDonald, 1989). In addition, removing a male song sparrow from his territory results in a new male taking over the territory, however, territory take-over can be prevented by playing the original male’s song from a loudspeaker (Nowicki et al., 1998). Also, in many species, playing recorded conspecific song within a male’s territory results in an aggressive response from the resident male including a large increase in song rate (Wingfield, 1984). There is also evidence that particular singing behaviours may be associated with aggressive interactions between males, including song-type matching (responding to a rival by singing the same song type the rival sang), frequency matching (adjusting the frequency of song to match the rivals), and singing at a low amplitude (Searcy and Beecher, 2009).

Song also attracts and stimulates females. There are many features of song that females may attend to when choosing a mate (Nowicki et al., 2002a; Searcy and Yasukawa, 1996). These features can be organized into two broad categories: song performance (how a bird sings) and song structure or composition (what a bird sings). First, features of song performance can include song output, which is typically defined as the length of song bouts or the rate of singing, with females often showing a preference...
for male’s with greater song output. For example, male European starlings that sing
longer song bouts pair earlier in the breeding season, acquire more mates in the wild, and
are preferred by females in laboratory experiments (Eens et al., 1991). Another aspect of
song performance that can affect female preference is the ability to produce physically
challenging notes such as trills that require rapid and precise coordination of vocal tract
movement (Podos, 1997). For example, female swamp sparrows perform more
copulation displays to songs containing trills that are more challenging to produce (trills
that contain syllables that are both repeated at a fast rate and encompass a large range of
frequencies; Ballentine et al., 2004). Lastly, females may also prefer males that produce
songs or syllables with a greater degree of stereotypy (high consistency from one
rendition to the next). For example, male chestnut-sided warblers (Setophaga
pensylvanica) that sing more stereotyped song obtain more extra-pair copulations (Byers,
2006). Measures of song performance are typically considered dynamic traits and these
features of song can even show a great deal of within-individual variation in closed-
ended song learners whose repertoires are fixed in adulthood (Smith et al., 1997;
Sockman, 2009).

Second, in addition to song performance, females may show a preference based
on the structure and composition of a male’s song. Females may prefer males that sing
more complex song, which is typically defined as the number of song types or syllables
in their repertoire. For example, male red-winged blackbirds with larger song-type
repertoires obtain more mates (Yasukawa et al., 1980). Similarly, free-living male great
reed warblers (Acrocephalus arundinaceus) with larger repertoires acquire more extra-
pair copulations (Hasselquist et al., 1996). Females may also show a preference for songs
that more closely resemble the dialect of their natal population. In support of this, female white-crowned sparrows perform more copulation displays to recorded songs that match the dialect where they were hatched compared to songs of a foreign dialect (MacDougall-Shackleton et al., 2001). Lastly, females may prefer songs that more accurately resemble the tutor song from which they were copied. For example, captive female song sparrows perform more copulation displays to recorded songs that contain less invented material and to songs that more accurately match the tutor model from which they were copied (Nowicki et al., 2002b). Importantly, in closed-ended song learners, the structure and composition of a male’s song is a static trait that is fixed during development since males do not learn new songs in adulthood. Therefore, these song features are reflective of the quality or quantity of song learning (Nowicki et al., 2002a).

1.4 Evolution of female song preferences

Birdsong has long been studied as an example of a sexually selected trait because of its role in mate attraction (Podos et al., 2004; Searcy, 1986). Although it is clear that females show a preference for certain features of song, why females have this preference and how it evolved is less clear. Many hypotheses have been proposed to explain the evolution of sexually selected traits. Three hypotheses have been commonly applied to birdsong: 1) Fisherian or runaway selection, 2) sensory exploitation, and 3) honest indicator models.
Fisher’s model of runaway selection proposes that female preference for a trait might spread because females with the preference would have sons that both express the trait and have alleles for the preference (Fisher, 1930). These sons would have a mating advantage because they possess the trait and over time both the trait and female preference would become more prevalent. The intensity of the preference will continue to evolve so long as the sons of females with the preference have a mating advantage over the sons of females who do not. Thus females with the preference gain indirect benefits because their sons are favoured in the next generation. To date, there is little evidence to support models of runaway selection when applied to birdsong (Nowicki and Searcy, 2005; Searcy, 1986). Specifically, many features of song do not show the exaggeration that would be predicted by the runaway selection model (Nowicki and Searcy, 2005). In addition, this model requires there to be genes for both the trait and female preference. Few studies have examined the heritability of song or female preference for song. One study found high heritability for unlearned vocalizations in zebra finches, but low heritability for learned features of song such as syllable repertoire size (Forstmeier et al., 2009).

Sensory exploitation models propose that female preference for a trait might evolve if males exploit a preexisting sensory or perceptual bias of the females (Endler and Basolo, 1998). In this hypothesis, features of the female’s nervous system may predispose her to respond more strongly to certain male characteristics. There would be no benefit to the female choosing males in this way but males with the trait would possess a mating advantage. There is some evidence that females may have a preexisting bias for larger song repertoires (Collins, 1999). For example, female common grackles
perform more copulation displays to repertoires containing four song types over repertoires containing one song type, even though male grackles only sing one song each (Searcy, 1992). This suggests that female grackles may have a preexisting bias to respond preferentially to multiple song types, potentially because multiple song types reduce habituation in the listener (MacDougall-Shackleton, 1997). However, sensory exploitation models are unlikely to explain female preference for other song features such as geographic variants or highly stereotyped song (Nowicki and Searcy, 2005).

Honest indicator models of sexual selection propose that female preference for a trait evolved because the trait indicates some aspect of male quality, thus providing benefits to females with the preference. These hypotheses stem from Zahavi’s handicap model of sexual selection, which posits that elaborate male traits are costly to produce or maintain such that they might decrease male survivorship (Zahavi, 1975). This cost functions as a *handicap* and only males who are of high quality will be able to express the trait at high levels. Thus, the cost incurred by the elaborate trait ensures its honesty in indicating the quality of the signaler (Zahavi, 1975). In this case, a female could gain *direct* benefits from mating with a high quality male, for example, by gaining access to more resources, as well as *indirect* benefits in the form of “good genes” being passed on to her offspring. Although the concept of good genes is often vague, in general, indicator models of sexual selection have received a great deal of empirical support (Hill, 1991; Knapp and Kovach, 1991), including when applied to birdsong (reviewed in Nowicki et al., 2002a). A key component of this hypothesis is that the male trait must reflect some aspect(s) of quality or condition of the male. Indeed, consistent with indicator models of sexual selection, many features of song are correlated with measures of male quality or
condition. For example, in free-living song sparrows, song complexity is correlated with regulation of the hypothalamic-pituitary-adrenal (HPA) axis (MacDougall-Shackleton et al., 2009; Schmidt et al., 2012), body condition (Pfaff et al., 2007), immune function (Reid et al., 2005), and ability to defend a territory (Hiebert et al., 1989). Syllable repertoire size also predicts male parental effort in sedge warblers (Buchanan and Catchpole, 2000). In European starlings, song bout length is positively correlated with humoral immunity (Duffy and Ball, 2002) and performance on a spatial foraging task (Farrell et al., 2011). Thus it appears that song may honestly indicate several aspects of male quality to females.

1.5 The costs of song: The Developmental Stress Hypothesis

Although there is a great deal of evidence to suggest that song serves as an honest indicator of male quality (Nowicki et al., 2002a), one problem with this hypothesis is that it has been difficult to determine the costs associated with some features of birdsong. Costs in the production or maintenance of a signal are necessary in order for the trait to honestly reflect the quality of the signaler, otherwise the signaler will benefit by exaggerating the trait so that they appear to be of higher quality. The costs of some features of song, such as song output, are more obvious. A male who sings more might make himself more vulnerable to predation, or singing may take time away from other vital activities such as foraging for food. However, the costs of other features, such as song complexity or singing a song that resembles a tutor’s song, are less clear. Nowicki
and colleagues (1998, 2002) proposed the Developmental Stress Hypothesis (originally called the Nutritional Stress Hypothesis) to explain the costs of such features of song. This theory proposes that the costs are not in the production or maintenance of the signal, but rather in its development. Specifically, song is learnt early in life during the same time that the song-control system is developing. During this time, altricial songbirds are likely to be exposed to a variety of environmental stressors, especially nutritional stress since they are entirely dependent on their parents for food. This hypothesis proposes that individuals who experience fewer stressors early in life, or are more resistant to stressors, will experience superior neural development and consequently produce the highest quality songs in adulthood. In this way, the learned features of song serve as reliable indicators of a male’s early ontogeny and his subsequent adult phenotype. Therefore, a female choosing a male based on the learned features of song may gain both direct benefits from mating with a phenotypically superior mate (e.g. access to a better territory) as well as indirect benefits (e.g. genes allowing for more developmental stability passed on to her offspring; Nowicki et al., 1998; Nowicki et al., 2002a).

A key prediction of the Developmental Stress Hypothesis is that early-life stress should affect development of the song-control system and adult song production. To date there is substantial evidence to support this prediction (reviewed in MacDougall-Shackleton and Spencer, 2012). For example, zebra finches exposed to food restriction or treated with the glucocorticoid hormone corticosterone (CORT) early in life had smaller volumes of HVC in adulthood (Buchanan et al., 2004) and also produced songs that were shorter and contained fewer syllables (Spencer et al., 2003). However, another study of zebra finches found no effect of food restriction on syllable repertoire size, although food
restriction decreased the accuracy of song learning (Brumm et al., 2009). Experimentally increasing the natal brood size of zebra finch nestlings (which presumably exposes them to more stress) did not affect syllable repertoire size in adulthood (Holveck et al., 2008), but has been shown to decrease song syntax learning accuracy (Holveck et al., 2008) and song rate (de Kogel and Prijs, 1996). European starlings exposed to an unpredictable food supply in the fledgling period have been shown to produce shorter and fewer song bouts (Buchanan et al., 2003) and to have smaller song repertoires (Spencer et al., 2004). In addition, swamp sparrows exposed to food restriction during development had smaller volumes of RA relative to the size of the telencephalon and reduced song learning accuracy in adulthood (Nowicki et al., 2002a). In this study, food restriction had no effect on the number of song types a male sang, but swamp sparrows have small song repertoires (only 2–4 unique songs) with little variation between males in a population, which would make it difficult to detect an effect of stress on song repertoire size (Nowicki et al., 2002a). Thus, it appears that early-life stress may affect development of the song-control system and several features of adult song production.

### 1.6 Developmental indicators as sexually selected traits

In addition to song, early-life stress may have long-term effects on many other physiological and behavioural traits (Harris and Seckl, 2011). For example, in songbirds, early-life stress may affect the immune system (Buchanan et al., 2003), regulation of the HPA axis (Pravosudov and Kitaysky, 2006), and spatial learning (Farrell et al., 2011). As mentioned above, song has been shown to correlate with several behavioural and
physiological traits in adulthood (Nowicki et al., 2002a). One hypothesis to explain how song comes to be correlated with these functionally independent traits in adulthood is that the period of song acquisition may overlap with the development of these other traits and both song and these other traits may be affected by early-life stressors, that is they might be developmentally correlated traits (Fig. 1-3; Spencer and MacDougall-Shackleton, 2011). In this way, song may serve as a reliable indicator of a male’s early ontogeny and subsequent adult phenotype.

**Figure 1-3** Correlations amongst functionally independent traits in adulthood (indicated by double arrows at time point D) might arise because stressors occurring at different time points may affect the development of many traits simultaneously. In this example, a stressor occurring at time point A will have long-term effects on song, the hypothalamic-pituitary-adrenal (HPA) axis, and other traits, causing correlations among these traits in adulthood. Stressors at time point B affect only song and other traits, while stressors at time point C affect only song and the HPA axis. Modified from Spencer and MacDougall-Shackleton, 2011.
The idea that a sexually selected trait may serve as a reliable indicator of development is not unique to birdsong. Indeed, any sexually selected trait that is susceptible to early-life stress and that develops simultaneously with other physiological or behavioral traits may provide information about the quality of an individual’s development to potential mates or rivals (Spencer and MacDougall-Shackleton, 2011). For example, fluctuating asymmetry (FA; small deviations from symmetry in bilaterally symmetric traits) of visual or morphological traits may be involved in mate choice (Moller, 1992; Thornhill and Gangestad, 1994) and is affected by a variety of environmental stressors including nutrient restriction, extreme temperatures, and exposure to parasites and pathogens (Parsons, 1990). In addition, in humans, body and facial FA have been shown to predict disease resistance (Thornhill and Gangestad, 2006) and performance on cognitive tests (Furlow et al., 1997). Cognition (de Rooij et al., 2010) and immune function (McDade et al., 2001) are also affected by early-life stress in humans, raising the hypothesis that FA, immune function, and cognition might be developmentally correlated traits in humans. Plumage colouration is another trait that might serve as a developmental indicator. For example, melanin-based plumage is a sexually selected trait in barn owls (*Tyto alba* [Roulin, 1999]). Nestling barn owls treated with CORT develop lighter coloured feathers (Roulin et al., 2008), suggesting that early-life stress may affect plumage coloration. Plumage coloration may also predict disease resistance in this species (Roulin et al., 2001). In addition, CORT treatment has been found to suppress immune function in barn owl nestlings (Stier et al., 2009). Therefore, immune function and melanin-based plumage coloration may be developmentally
correlated traits in barn owls. Thus, in addition to FA and plumage, birdsong may be one example of a general process whereby indicators of development come to be sexually selected traits (Spencer and MacDougall-Shackleton, 2011).

1.7 Thesis objectives

The goal of this thesis was to provide an experimental test of the Developmental Stress Hypothesis in song sparrows. Although evidence in support of this hypothesis has accumulated, some outstanding issues remain, which I addressed by pursuing three primary research objectives:

My first objective was to determine the effects of early-life stress on a variety of physiological traits in adulthood that might be important components of phenotypic quality. A key prediction of the Developmental Stress Hypothesis is that stressors experienced during development should affect both adult male song production and phenotypic quality, including traits that might affect the direct benefits received by female mates (Nowicki and Searcy, 2005). However, whether or not stressors that affect adult song production also have widespread effects on other physiological traits remains relatively unexplored. I addressed this issue in the following three chapters by determining if early-life stress has long term effects on body size, body composition, and metabolic rates in Chapter 2, the innate and acquired immune system in Chapter 3, and regulation of two endocrine systems, the HPA axis and the hypothalamic-pituitary-gonadal axis in Chapter 4. Many of these physiological and morphological traits relate to
In addition, because these studies were conducted on both male and female song sparrows, I also determined whether there were sex differences in the effects of early-life stress on these traits.

My second objective was to determine the long-term effects of early-life stress on adult song production and the song-control system in male song sparrows. As reviewed above, there is evidence from several studies that developmental stress does affect adult song production. However, most of these studies have been conducted on species that sing only one or a few unique song types (e.g. zebra finch, swamp sparrow, Bengalese finch [Lonchura striata domestica]). Therefore, it has been difficult to determine if early-life stress has long-term effects on song repertoire size. However, this question can be easily addressed in song sparrows because they have relatively complex repertoires and sing 5-12 unique song-types (Pfaff et al., 2007). In addition, most studies conducted to date have focused on song complexity and song learning accuracy and little is known about how early-life stress affects measures of vocal performance (Podos et al., 2009). Therefore, in Chapter 5, I determined the effects of early-life stress on multiple song features including song complexity (number of song types and syllables in a male’s repertoire), song learning accuracy, and measures of vocal performance not previously tested including song stereotypy and trill production. In addition, in Chapter 5, I also determined the long-term effects of early-life stress on the song-control system including the volumes of RA, HVC, and area X, as well as the number of neurons in HVC.

My third objective was to determine if early-life stress affects the behavioural response of female song sparrows to song. So far the vast majority of studies conducted
on songbirds have focused exclusively on male song production and little is known about how early-life stress influences female song preferences. Therefore, in Chapter 6, I determined the long-term effect of early-life stress on the behavioural response of female song sparrows to song. In addition, I determined the effect of early-life stress on the brain of female song sparrows including the volume of HVC and RA and the induction of the immediate-early gene Zenk (an acronym for Zif-268, Egr-1, NGFIA, and Krox-24) in auditory forebrain regions following exposure to song presentation.
1.8 References


Chapter 2

2 Developmental Stress has Sex-Specific effects on Nestling Growth and Adult Metabolic Rates but no effect on Adult Body Size or Body Composition in Song Sparrows

2.1 Introduction

Variation in the prenatal and postnatal environments can lead to long-term variation in adult phenotype, a process often referred to as developmental programming (McMillen and Robinson, 2005). In particular, exposure to stressors early in life, such as nutritional restriction, infection, or elevated glucocorticoid levels, can alter development leading to permanent changes in physiology (McMillen and Robinson, 2005; Rinaudo and Wang, 2012; Welberg and Seckl, 2001). In humans, these early-life events alter fetal or infant growth and may predispose individuals to disease, especially those involving energy metabolism. For example, low birth weight in humans is associated with increased risk of obesity, type II diabetes, and impaired lipid metabolism in adulthood (Barker et al., 1993; Rinaudo and Wang, 2012). Individuals exposed to famine in utero have higher indices of obesity (Ravelli et al., 1999) and impaired glucose tolerance (Ravelli et al., 1998), suggesting that nutritional restriction during development may be a particularly important risk factor for disease in later life. In support of this, rats exposed to a low protein diet in utero or during the early postnatal period exhibit altered postnatal growth
and long-term changes in glucose metabolism and insulin resistance (Zambrano et al., 2006). In mammals, the specific physiological effects of a stressor often depend on the stage of development in which exposure occurred (Painter et al., 2005).

In addition to changes in energy metabolism, studies in birds have shown important links between variation in the early rearing environment and variation in metabolic rates. For example, zebra finches (Taeniopygia guttata) raised in experimentally enlarged broods had higher standard metabolic rates (SMRs) in adulthood compared with those raised in smaller broods (Verhulst et al., 2006). In the same species, treatment with the glucocorticoid hormone corticosterone (CORT) during the nestling period increased overnight variability in SMRs; however, this effect was seen only during the treatment period and not in adulthood (Spencer and Verhulst, 2008). In both of these studies, the effect of the stressor on metabolic rates was more severe in females than in males, suggesting that early-life stressors could have sex-specific programming effects on energy expenditure. Variation in metabolic rates could in turn have important fitness consequences. For example, individuals with higher metabolic rates have higher energy requirements and may have to spend more time foraging for food or be less likely to survive food shortages. High resting metabolic rates have also been linked to decreased longevity (Manini, 2010; Speakman, 2005). In addition, basal metabolic rates (BMRs) are positively correlated to reproduction, such that species with high BMRs often have higher reproductive rates (Hennemann, 1983). Therefore, at the inter-specific level, variation in metabolic rates may mediate important tradeoffs between reproduction and survival. However, whether or not variation in metabolic rates is related to reproduction and survival within a species is less clear.
The physiological mechanisms underlying the effects of early-life stressors on energy metabolism and metabolic rates involve many processes (Rinaudo and Wang, 2012). The stressor may directly alter the development of an organ, resulting in permanent changes in organ morphology or function. For example, prenatal and postnatal protein restriction in rats reduces the growth of the pancreas, spleen, muscle, and liver (Desai et al., 1996). Changes in organ size could be due to reductions in cell number or cell size. In rats, early-life protein restriction decreases beta cell proliferation and the size of islets in the pancreas (Snoeck et al., 1990). A variety of stressors may also increase fetal or neonatal glucocorticoid exposure, which affects offspring growth and development (Fernandez-Twinn and Ozanne, 2006; Welberg and Seckl, 2001). Food restriction can increase baseline and stress-induced glucocorticoid levels in birds (Kempster et al., 2007; Kitaysky et al., 2001a), amphibians (Crespi and Denver, 2005), and mammals (Lesage et al., 2001). In turn, early-life glucocorticoid exposure has many of the same detrimental effects as nutritional restriction, including growth retardation (Spencer et al., 2003), impaired brain development (Buchanan et al., 2004), and altered energy metabolism (Harris and Seckl, 2011; O'Regan et al., 2004). In addition, stressors during development can alter typical patterns of somatic growth, which can also be detrimental. A stressor may initially retard growth but be followed by a period of rapid growth acceleration once the stressor subsides (catch-up growth) such that there are no long-term effects on body size. Although beneficial in the short-term, catch-up growth may negatively affect health and fitness (Hales and Ozanne, 2003; Metcalfe and Monaghan, 2001). For example, catch-up growth results in long-term increases in resting
metabolic rates in zebra finches (Criscuolo et al., 2008) and decreases longevity in rats (Jennings et al., 1999).

I examined the effects of early-life food restriction and treatment with exogenous CORT on: 1) nestling growth, 2) adult body size, 3) adult body composition, and 4) adult metabolic rates in song sparrows (Melospiza melodia). I used CORT treatment to determine whether glucocorticoids have effects similar to those of food restriction on growth and physiology. Because a variety of stressors increase glucocorticoid levels, this allowed me to determine whether a number of different stressors might affect growth and metabolism via CORT in song sparrows. I monitored nestling growth during and after the treatment period to determine whether birds exhibited catch-up growth and to evaluate the long-term effects of each treatment on adult body size. I also used quantitative magnetic resonance (QMR) analysis to examine body composition, to determine whether developmental stress has long-term effects on lean and fat mass. Last, I investigated the effects of food restriction and CORT treatment on metabolic rates, specifically SMRs and peak metabolic rates (PMRs). Although past studies on birds have examined the effects of variation in the early rearing environment on SMRs, no studies have examined PMR to determine whether early-life stress could affect the ability of an animal to perform intense exercise. Because the ability to perform intense exercise might be necessary for birds to forage, escape predators, and complete annual migrations, changes in PMR could have important fitness consequences. I predicted that exposure to early-life food restriction or CORT treatment would result in reduced nestling growth, lowered adult total and lean body mass, increased SMR, and decreased PMR.
2.2 Methods

2.2.1 Study subjects and rearing conditions

Song sparrow nests were located near Newboro, Ontario, Canada (44°38’N, 76°20’W), during May and June 2010. Nests were monitored to determine the day-of-hatch. All nests hatched between May 9\textsuperscript{th} and June 7\textsuperscript{th} 2010 and represented the first brood for the pair that year. The territorial male associated with each nest was caught using mistnets and conspecific song playback, and morphological measurements were collected from these males (see below) prior to broods hatching, in April and May 2010. Because extra-pair paternity is infrequent in this study population [consistently below 10% of nestlings (Potvin and MacDougall-Shackleton, 2009; E.A.M.-S., unpublished data)], the resident male was presumed to be the genetic father of nestlings hatching on the territory. I did not catch the female associated with each territory (the presumed mother) because I did not want to interfere with egg laying or incubation, which may increase the chance of nest predation or desertion. A total of 47 nestlings from 15 broods were used for this study. Of these, 43 were brought into captivity at 3-4 days post-hatch (d3-d4), and 4 were brought in at ~d7 (mean=3.44 days, SEM=0.16; Table 2-1).

Nestlings were kept warm using heat lamps and electric heating pads until they developed feathers (~d7), and were transported to the University of Western Ontario, London, Ontario, Canada and housed at the Advanced Facility for Avian Research for the remainder of the experiment. Nestlings were housed in a cage with their siblings until they began eating independently (~d25), at which point they were housed individually. Birds were kept on a long day photoperiod (16L:8D) until August 16th, 2010, and then
switched to short days (10L:14D) for the remainder of the experiment. Sex of nestlings was determined using polymerase chain reaction (PCR) amplification of genes on the sex chromosomes (Griffiths et al., 1998). Amplification and electrophoresis conditions are described elsewhere (Potvin and MacDougall-Shackleton, 2010).

Table 2-1 Age and mass of song sparrow nestlings at the start of the experiment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Food Restriction</th>
<th>CORT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Sample Size</td>
<td>9</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Age at Capture (d)</td>
<td>3.56±0.44</td>
<td>3.71±0.57</td>
<td>3.25±0.16</td>
</tr>
<tr>
<td>Mass at Capture (g)</td>
<td>8.98±1.18</td>
<td>9.45±1.13</td>
<td>9.60±0.69</td>
</tr>
</tbody>
</table>

Note: Age at capture and mass at capture represent the age and mass of nestlings the day they were brought into captivity. Values are means ± SEM. CORT = corticosterone

2.2.2 Experimental treatments

Within each brood, nestlings were assigned to one of the three treatment groups: control (ad libitum food), food restriction or CORT treatment. This was done using block randomization, such that if there were three or more nestlings in a brood, at least one nestling was assigned to each treatment. This method of randomization was used instead of true randomization to ensure that I had similar sample sizes for each treatment group. In addition, this procedure allowed me to ensure that there were never more than two
nestlings from a given brood in a treatment, therefore allowing me to control for nest of origin as best as possible. In total, there were 16 control subjects (9 males, 7 females), 16 food-restricted subjects (8 males, 8 females) and 15 CORT-treated subjects (6 males, 9 females; Table 1). Food restriction and CORT treatment lasted from d7 to d60 (see Fig 2-1 for timeline).

All nestlings received a standard hand-rearing diet administered via 1mL syringes. The diet consisted of ground Mazuri Small Bird Maintenance diet (catalogue number 56A6, Brentwood, MO, USA), hard-boiled chicken eggs (shells removed), wheat germ, water, and Prime avian vitamin supplement (Rolf C. Hagen Inc, Montreal, QC, Canada). I followed a food restriction protocol that has been used for a variety of songbird species (Nowicki et al., 2002; MacDonald et al., 2006). Briefly, for each brood, the control and CORT-treated birds were first fed ad libitum. I calculated the average amount of food eaten by nestlings in these two groups and then fed 65% of this amount to the food-restricted siblings. Nestlings were fed every 30 min during daylight hours until d18. At this time, I added food dishes to the cages and slowly lengthened the feeding interval to encourage birds to eat independently. Once feeding independently, birds were fed a 50:50 mix of ground Mazuri Small Bird Maintenance Diet (catalogue number 56A6) and premium budgie seed (Rolf C. Hagen). To continue the food restriction stressor into the fledgling period, I removed food cups for 3 h per day until d60 for this treatment group. The start of this 3 h period was randomized each day. This protocol has been used in European starlings and affects adult body size, immune function, song production, and spatial learning (Buchanan et al., 2003; Farrell et al., 2011).
Figure 2-1 Experimental timeline used to determine the effects of early-life food restriction or corticosterone treatment on nestling growth and adult body size, body composition, and metabolic rates in song sparrows.

For CORT treatment, CORT was dissolved in peanut oil and administered orally to birds. This non-invasive technique results in a transient increase in CORT similar to that experienced in response to an acute stressor, and in nestling zebra finches affects nestling growth, brain development, and song learning (Buchanan et al., 2004; Spencer et al., 2003). I used a dose of 0.87 µg/g body mass, which was determined during pilot studies (see below). CORT was fed to nestlings twice per day, once in the morning and once in the evening. Control and food-restricted birds were fed peanut oil alone. Once birds were eating independently, CORT was first injected into wax worm larvae and then fed to birds once per day in the morning until d60 (Breuner et al., 1998). Control and food-restricted birds were fed wax worm larvae injected with oil only.

I conducted a pilot study to verify that orally administering CORT resulted in a transient increase in CORT similar to that observed in song sparrows in response to restraint stress (MacDougall-Shackleton et al., 2009; Newman et al., 2008). I injected CORT into wax worm larvae (dose = 1 µg/g body mass) and fed the worms to captive song sparrows. Blood samples were collected 0, 10, or 30 min post-ingestion of the
worm. CORT levels were low 0 min post-ingestion (n=4, 4.16 ± 2.38 ng/mL), peaked 10 min post-ingestion (n=3, 173.13 ± 51.40 ng/mL) and had begun to decrease after 30 min (n=4, 61.58 ± 9.35 ng/mL). Because peak CORT levels were slightly higher than CORT levels post-restraint in my study population (MacDougall-Shackleton et al., 2009; Schmidt et al., 2012), I used a slightly lower dose of 0.87 µg/g body mass for my experiment. In studies using a similar manipulation in white-crowned sparrows, CORT levels peaked 7 min post-ingestion of the worm, were still elevated 30 min post-ingestion, and had returned to baseline after 60 min (Breuner et al., 1998). Therefore, this method of administration results in a transient increase in CORT that is very similar to the increase observed after exposure to an acute stressor.

To verify that the CORT treatment was effective during the experiment, I collected blood samples (~30 µL) on d10 and d45, 10 min after administration of CORT or vehicle to determine plasma CORT levels. CORT was quantified in unextracted plasma using a radioimmunoassay (07-120103, MP Biomedicals, Sant-Ana, CA, USA) that has been previously validated in song sparrows (Newman et al., 2008). Three separate assays were conducted and samples from all subjects were randomly assigned to an assay such that each treatment was equally represented in each assay. The lower limit of detectability ranged from 1.8 – 2.6 ng/mL. Inter-assay variation was 5.5% for a low control (39 ng/mL) and 4.1% for a high control (179 ng/mL). Intra-assay variation was 9.4% for the low control and 3.9% for the high control.
2.2.3 Body measurements

Body mass was measured using a spring scale to the nearest 0.1 g. I measured nestling body mass daily as soon as the lights came on (5:30 AM) until d25. Thereafter, I measured body mass every 5 days until d60. Adult body mass (at ~ 7 months) was measured the evening prior to and the morning following SMR measurements and prior to PMR measurements. To compare adult masses across treatments, I used masses recorded the morning after SMR measurements when birds were in the post-absorptive state. I also measured the length of the wing chord and tarsus to the nearest 0.1 mm using dial calipers on d25, d45 and during adulthood prior to SMR measurements.

2.2.4 Body composition analysis

I determined lean and fat mass using quantitative magnetic resonance (QMR) analysis (Guglielmo et al., 2011) the morning following SMR determination when birds were still in the post-absorptive state. The QMR unit (Echo-MRI-B, Echo Medical Systems, Houston, TX, USA) was custom-designed for use with small birds and bats. The QMR was calibrated daily using 5 g and 94 g canola oil standards. To use the QMR, awake birds were placed into plastic holding tubes and inserted into the QMR analyzer and scanned using the “small bird” and “two accumulation” settings of the Echo MRI software. Fat and lean mass measurements were reported to the nearest 0.001 g. Fat and lean mass measurements were slightly adjusted to improve accuracy using calibration equations developed from house sparrows and zebra finches [fat mass: raw value x 0.94; lean mass: raw value x 1.021 (Gerson and Guglielmo, 2011; Guglielmo et al., 2011)]. A validation study conducted previously showed that the coefficients of variation for fat and
lean mass are 3% and 0.5%, respectively, and relative accuracies are ±11% and ±1%, respectively (Guglielmo et al., 2011).

2.2.5 Respirometry

Standard metabolic rates

Metabolic rates were measured using open-circuit respirometry. I measured the SMR of birds between December 2010 and January 2011 when birds were ~7 months old (mean=214 days, SEM=0.88), which was about 5 months after the end of the stress treatments. Beginning at 20:00 h, body measurements were taken and birds were placed into one of 5 stainless-steel chambers. Chambers were placed in a temperature-controlled cabinet at 30°C, which is within the thermoneutral zone for other species of songbirds that are similar in size to song sparrows (Root et al., 1991). Four birds were individually placed into the chambers every night and the remaining chamber was used for baseline measurements. Birds fasted in the chambers for 3 h and then O\textsubscript{2} consumption was measured in the remaining 9 h of the overnight period. Thus measurements were taken during the inactive period, in the post-absorptive state, and while birds were housed on short-days and thus in non-breeding condition. However, the exact temperature range of the thermoneutral zone for song sparrows is unknown so I refer to my measurements as SMR instead of BMR. Incurrent air was scrubbed of CO\textsubscript{2} and water vapor using soda lime and Drierite (W.A. Hammond Drierite Company Ltd., Xenia, OH, USA), respectively. The five sealed chambers received a constant flow of 450 mL/min. Excurrent air was sub-sampled at 150 mL/min and passed through a Drierite column to the CO\textsubscript{2} analyzer (catalogue number: CA-2A; Sable Systems Las Vegas, NV, USA) and
the O₂ analyzer (Sable Systems FC-1B), with CO₂ and water scrubbing between the two
gas analyzers. Gas analyzers were calibrated daily using a standard containing 20.9% O₂
and 2% CO₂ balanced with N₂ (Praxair, London, ON, Canada). Using a multiplexer
(Sable Systems), one chamber was measured at a time for 10 minutes before switching to
the next chamber. In total, each bird was measured 12 times throughout the night for 10
minutes at a time. All instruments were connected to an analog-to-digital converter (UI-2
model, Sable Systems), which was connected to a laptop computer. Data analysis was
completed using Warthog Systems Lab Analyst software (M.A. Chappel, University of
California Riverside, Riverside, CA, USA). SMR values reported were calculated as the
minimum 10 min mean of O₂ consumption throughout the measurement period. I
calculated the rate of O₂ uptake (VO₂) [based on eqn 10.6 in Lighton (Lighton, 2008)]
and converted VO₂ to watts (W). The equation that I used to calculate VO₂ used the data
for both O₂ consumption and CO₂ production (Lighton, 2008). The following morning,
birds were weighed, analyzed for body composition using QMR, and returned to their
home cage.

*Peak metabolic rates*

The same flow system used to determine SMRs was used to determine the PMR
of each bird. After measuring SMR, birds were left undisturbed in their home cage for
one full day. I measured PMR the afternoon of the following day (39-42 h after the start
of SMR measurement). PMR was measured using an enclosed running wheel modified
for use with flying birds (Pierce et al., 2005; Price and Guglielmo, 2009). The wheel
(width=16 cm; diameter=24 cm) was made of acrylic plastic and was lined with rubber.
Three ping pong balls were placed in the wheel to prevent birds from walking. Air flowed
into the wheel at a rate of 4 L/min and was sub-sampled as described above for measurements of SMR. Food dishes were removed 3 h before testing to ensure that birds were in the post-absorptive state. Beginning at 11:00, and no later than 14:00, birds were weighed and placed into the flight wheel. The flight wheel was covered and birds were allowed to acclimate for 10 min. The wheel was then spun manually to initiate exercise. The wheel was kept in constant motion so that birds were forced to hop and hover until PMR was reached (always occurred within 15 min). This method provides a significant aerobic challenge and has been used to estimate PMR in previous studies of flying birds (Pierce et al., 2005; Price and Guglielmo, 2009). In all cases, after PMR was reached O$_2$ consumption decreased and then stabilized. The PMR of an individual was calculated as the maximum mean of O$_2$ consumption over a 1 min period. Data are expressed as watts and I calculated the metabolic scope of each individual (PMR/SMR), which provides an estimate of intensity of exercise (Pierce et al., 2005).

2.2.6 Data analysis

Statistical analyses were conducted using SPSS version 19 (IBM, Armonk, NY, USA). For CORT levels, I conducted linear mixed models using restricted maximum likelihood (REML) models. Subject identity was added as a random factor with unstructured covariance. Age, treatment, and sex were included as fixed effects. Significant main effects of treatment were analyzed using least significant difference (LSD) pairwise comparisons.

I also used linear mixed models to analyze nestling growth data. I conducted two separate analyses to reflect the two different parts of the treatment period. The first
analysis involved the mass of nestlings from d9 to d18, that is, throughout the hand-rearing period. I expected the treatments to most strongly affect growth during this period because this is when the food restriction stressor was most severe and was also when CORT-treated birds were fed CORT twice per day instead of once. The second analysis involved the mass of nestlings from d19 to d60, the period in which birds began feeding independently up to the end of the treatment period. For both analyses, age was added as a repeated factor with first-order autoregressive covariance structure (West, 2009). Sex, treatment, and age were added as fixed effects. Significant sex x treatment interactions were further analyzed by conducting linear mixed models for each sex with treatment and age as fixed factors. Significant main effects of treatment were analyzed using LSD pairwise comparisons. Paternal body mass and hatch date were included as covariates and nest identity (the natal brood nestlings came from) was included as a random factor. The mass of nestlings the day they were brought into captivity, and thus before the treatments begun, was also included as a covariate in order to control for chance variation in mass or condition. One initial model was conducted for each age period (d9-d18 and d19-d60) that included the fixed factors (treatment, sex, age), the random factor (nest identity) and the covariates (hatch date, paternal mass, initial nestling mass). If the covariates or random factor were not significant they were removed from the analysis in order to create the simplest model possible.

To compare the effects of the treatments on body size, I analyzed mass, tarsus length, and wing length using a principal component analysis (PCA) at each age (d25, d45, adulthood), as these three measures were highly correlated. Data were log transformed before being entered into the PCA. At all three ages, the PCA revealed one
component with an eigenvalue greater than 1 (Table 2-2). I interpreted this component as representing overall body size. The resulting PC scores were then analyzed using two-way ANOVAs with treatment and sex as between-subjects factors. Significant main effects of treatment were compared using LSD pairwise comparisons. Hatch date was included as a covariate and nest identity was included as a random factor. At each age, the initial model included the fixed factors (treatment and sex), the random factor (nest identity) and the covariate (hatch date). If the covariate or random factor were not significant, they were removed from the analysis.

Body composition (fat, lean mass, adult mass) and metabolic rates (SMR, PMR, metabolic scope), were analyzed using two-way ANOVAs with sex and treatment as between-subjects factors. Significant sex x treatment interactions were further analyzed by conducting ANOVAs for each sex with treatment as a fixed factor. Significant main effects of treatment were analyzed using LSD pairwise comparisons. Hatch date was added as a covariate and nest identity as a random factor for analyses of both metabolic rates and body composition, and body mass was included as a covariate for analyses of metabolic rates. The initial models included the fixed factors (treatment and sex), the random factor (nest identity) and the covariates (hatch date, body mass). If the covariates or random factor were not significant, they were removed from the analysis.

Finally, total adult body mass and lean body mass of the hand-raised birds was directly compared with the mass of their fathers using simple linear regressions. All tests were two-tailed and were considered significant at $p \leq 0.05$. Data are presented as means ±SEM, adjusted for significant covariates and random factors where applicable.
Table 2-2 Principal component analysis for morphological measurements of song sparrows

<table>
<thead>
<tr>
<th>PC1</th>
<th>Eigenvalue</th>
<th>% Variance Explained</th>
<th>Factor Loadings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mass</td>
</tr>
<tr>
<td>day 25 PC1</td>
<td>1.71</td>
<td>56.86</td>
<td>0.83</td>
</tr>
<tr>
<td>day 45 PC1</td>
<td>1.71</td>
<td>56.89</td>
<td>0.77</td>
</tr>
<tr>
<td>Adult PC1</td>
<td>1.83</td>
<td>61.02</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Note: At each age, principal component analyses revealed one principle component (PC) with an eigenvalue greater than one.

2.3 Results

2.3.1 CORT levels

The exogenous CORT treatment was effective in significantly elevating plasma CORT levels (main effect of treatment: $F_{2,41.77}=84.79$, $p<0.001$). CORT levels 10 min post-administration of CORT or vehicle were higher in CORT-treated birds ($d_{10}=136.64 \pm 15.64$; $d_{45}=143.35 \pm 14.48$) than controls ($d_{10}=6.76 \pm 1.70$; $d_{45}=18.88 \pm 3.69$; $p<0.001$) or food-restricted birds ($d_{10}=4.19 \pm 0.62$; $d_{45}=28.24 \pm 4.45$; $p<0.001$). Control and food-restricted birds did not differ significantly in plasma CORT levels ($p=0.71$). Therefore, my method of oral CORT administration was effective at increasing circulating CORT, and levels reached those typically observed in wild song sparrows.
subjected to an acute stressor (MacDougall-Shackleton et al., 2009; Schmidt et al., 2012). I also detected a significant main effect of age ($F_{1,42.11}=7.51$, $p=0.01$), as CORT levels were higher at d45 than at d10. No significant main effect of sex was detected ($F_{1,41.79}=1.06$, $p=0.31$), nor were any of the interaction terms significant ($p>0.40$ in all cases).

2.3.2 Nestling growth

To compare mass between nestlings at the start of the treatment period (d7), I conducted an ANOVA with treatment and sex as fixed factors. The main effect of treatment was not significant at d7 ($F_{2,41}=0.60$, $p=0.56$). Neither the main effect of sex ($F_{1,41}=2.86$, $p=0.10$) nor the treatment x sex interaction ($F_{2,41}=1.67$, $p=0.20$) were significant.

For the hand-rearing period (d9-d18), both the treatment x sex ($F_{2,40.07}=6.24$, $p=0.004$) and the age x sex ($F_{9,182.60}=2.12$, $p=0.03$) interactions were significant (Fig 2-2A and 2-2B). However, neither the treatment x sex x age nor the treatment x age interactions were significant ($p>0.66$ in both cases). The mass of nestlings prior to the treatment period was positively related to mass during the hand-rearing period ($F_{1,39.94}=7.19$, $p=0.01$, estimate of fixed effect=0.16, s.e.m.=0.06). To explore the treatment x sex interaction, I conducted linear mixed models for each sex with treatment and age as fixed factors. For males, the main effect of treatment was significant ($F_{2,19.02}=3.98$, $p=0.04$; Fig 2-2A): CORT-treated males weighed more than control (p=0.03) and food-restricted (p=0.02) males, but control and food-restricted males did not
Figure 2-2 The effect of food restriction (Food Res) or corticosterone (CORT) treatment on nestling growth rates in male (A) and female (B) song sparrows. Insets show mass of nestlings during the hand-rearing period (days 9 to 18) when treatments were most intense. The total treatment period lasted from 7 days of age to 60 days of age. *p<0.05.
differ (p=0.80). The mass of males prior to the treatment period was positively related to mass during the hand-rearing period (F_{1,18.98}=4.24, p=0.05, estimate of fixed effect=0.25, s.e.m.=0.12). For females, similar to males, the main effect of treatment was significant (F_{2,20.08}=4.58, p=0.02; Fig 2-2B). However, control females weighed more than both food-restricted (p=0.01) and CORT-treated (p=0.02) females. Food-restricted and CORT-treated females did not differ (p=0.81). The mass of females prior to the treatment period was positively related to mass during the hand-rearing period (F_{1,19.94}=4.24, p=0.05, estimate of fixed effect=0.11, s.e.m.=0.05).

The second analysis examined the latter part of the treatment period (d19-d60), after food cups had been added to cages and birds began to feed independently. During this period, neither the treatment x age x sex interaction, nor any of the two-way interactions were significant (p>0.10 in all cases). There was a significant main effect of sex (F_{1,37.75}=47.31, p<0.001); males were larger than females (Fig 2-2A and 2-2B). The main effect of age was also significant (F_{13,266.257}=4.87, p<0.001). The main effect of treatment was not significant (F_{2,37.78}=0.86, p=0.43). The mass of nestlings prior to the treatment period was positively related to the mass of nestlings during the latter part of the treatment period (F_{1,35.94}=4.55, p=0.04, estimate of fixed effect=0.10, s.e.m.=0.05). Hatch date was also positively related to mass during this period (F_{1,35.92}=4.67, p=0.04, estimate of fixed effect=0.05, s.e.m.=0.02). Finally, paternal body mass was also a significant covariate (F_{1,35.92}=4.04, p=0.05, estimate of fixed effect=0.26, s.e.m.=0.13); heavier fathers had heavier offspring.
2.3.3 Body size

On d25, after 18 days of experimental manipulation, the main effect of treatment on body size (PC scores) was not significant ($F_{2,41}=1.23$, $p=0.30$), nor was there a significant treatment x sex interaction ($F_{2,41}=0.22$, $p=0.80$). However, the main effect of sex was significant ($F_{1,41}=31.93$, $p<0.001$): males were larger than females (Fig 2-3A). On d45, after about 5 weeks of manipulation, the main effect of treatment on body size was significant ($F_{2,39}=3.53$, $p=0.04$): CORT-treated birds were smaller than control (p=0.02) and food-restricted birds (p=0.002), but control and food-restricted birds did not differ (p=0.37). Again, I observed a main effect of sex ($F_{2,39}=21.64$, $p<0.001$) such that males were larger than females (Fig 2-3B), but the treatment x sex interaction was not significant ($F_{2,39}=0.82$, $p=0.45$). Last, in adulthood, the main effects of treatment ($F_{2,27}=0.81$, $p=0.46$) and sex ($F_{1,27}=3.16$, $p=0.09$; Fig 2-3C) were not significant, nor was the treatment x sex interaction ($F_{2,27}=0.37$, $p=0.69$). Nest identity was significantly related to adult body size ($F_{14,27}=3.16$, $p=0.005$). Thus, the effects of my treatments on body size were limited to a period following rapid growth (d45) and were no longer apparent by adulthood.
The effect of food restriction or corticosterone (CORT) treatment on structural body size of song sparrows at (A) 25 days of age, (B) 45 days of age, and (C) in adulthood. Body size scores are the results from principal component analysis (PCA) that included measures of body mass, tarsus, and wing length. Results from the PCA can be found in Table 1. *p<0.05, **p<0.01.
2.3.4 Relationship with paternal mass

Despite the fact that the experimental treatments altered nestling growth, I observed no long-term effects on adult body size, suggesting that variation in final adult body size may primarily be due to heritable factors in song sparrows. To explore this possibility, I asked whether the adult mass of study subjects was related to the mass of their fathers. Paternal body mass was positively and significantly related to offspring body mass ($r^2=0.11$, $p=0.03$; Fig 2-4A) and lean mass ($r^2=0.23$, $p<0.001$; Fig 2-4B).

2.3.5 Body composition

For adult total body mass (Fig 2-5A), neither the main effects of treatment ($F_{2,27}=1.45$, $p=0.25$) or sex ($F_{1,27}=0.70$, $p=0.41$) were significant, nor was the treatment x sex interaction significant ($F_{2,27}=0.78$, $p=0.47$). Nest identity was significantly related to adult total body mass ($F_{14,27}=3.51$, $p=0.003$). For adult lean body mass (Fig 2-5B), there was no significant main effect of treatment ($F_{2,27}=1.50$, $p=0.24$). However, the main effect of sex was significant ($F_{1,27}=5.36$, $p=0.03$); males had a higher lean mass than females (Fig 2-5B). The treatment x sex interaction was not significant ($F_{2,27}=1.23$, $p=0.31$). Nest identity was significantly related to adult lean mass ($F_{14,27}=2.11$, $p=0.05$).

For adult fat mass (Fig 2-5C), the main effect of treatment was not significant ($F_{2,27}=1.20$, $p=0.32$). The main effect of sex was significant ($F_{1,27}=5.73$, $p=0.02$): females had a higher fat mass than males (Fig 2-5C). The treatment x sex interaction was not significant ($F_{2,27}=1.06$, $p=0.36$). Again, nest identity was significantly related to adult fat mass ($F_{14,27}=3.87$, $p=0.001$).
Figure 2-4 Simple linear regressions showing the relationship between paternal body mass and the (A) body mass and (B) lean mass of the experimental subjects in adulthood. The father was assumed to be the resident male bird on the territory where a nest was located, and was caught prior to hatching.
The effect of food restriction or corticosterone (CORT) treatment on (A) total body mass, (B) lean mass, and (C) fat mass. Treatments lasted from 7 days of age to 60 days of age. Body composition analysis was conducted using quantitative magnetic resonance analysis when birds were ~7 months of age. *p<0.05
2.3.6 Metabolic rates

For SMR (Fig 2-6A), body mass was a significant covariate ($F_{1,26}=26.13$, $p<0.001$) and nest identity was a significant random factor ($F_{14,26}=2.19$, $p=0.02$). The treatment x sex interaction was significant ($F_{2,26}=4.36$, $p=0.02$). To further analyze this interaction, I conducted ANOVAs for each sex with treatment as a fixed factor. For males, the main effect of treatment was not significant ($F_{2,8}=0.72$, $p=0.52$). For females, the main effect of treatment was significant ($F_{2,8}=5.81$, $p=0.03$). Control females had lower SMRs than food-restricted ($p=0.009$) and CORT-treated ($p=0.04$) females. The SMRs of food-restricted and CORT-treated females did not differ ($p=0.34$). For PMRs (Fig 6B), the main effects of treatment ($F_{2,26}=0.92$, $p=0.41$) and sex ($F_{1,26}=0.35$, $p=0.56$) were not significant, nor was the treatment x sex interaction ($F_{2,26}=0.14$, $p=0.87$). Nest identity was significantly related to PMR ($F_{14,27}=2.11$, $p=0.05$). For metabolic scope ((PMR/SMR), Fig 2-6C), the main effects of treatment ($F_{2,41}=0.88$, $p=0.42$) and sex ($F_{1,41}=1.26$, $p=0.27$) were not significant, nor was the treatment x sex interaction significant ($F_{2,41}=0.05$, $p=0.96$).
Figure 2-6 The effect of food restriction or corticosterone (CORT) treatment on (A) standard metabolic rates, (B) peak metabolic rates, and (C) metabolic scope of song sparrows. Treatments lasted from 7 days of age to 60 days of age. Metabolic rates were assessed when birds were ~7 months of age. *p<0.05, **p<0.01
2.4 Discussion

2.4.1 Food restriction affected growth and metabolic rates without increasing CORT

CORT levels did not differ between food-restricted and control subjects in my study. Therefore, food restriction might affect growth and metabolic rates independently of CORT, for example by directly altering organ morphology or cell number (Rinaudo and Wang, 2012). However, I cannot rule out the possibility that food restriction affects development by altering stress physiology. First, I only measured CORT levels at two ages (d10 and d45). It is possible that food restriction affected CORT levels during a time in the treatment period when blood samples were not collected. Second, I only measured baseline plasma CORT levels. In European starlings (Sturnus vulgaris), exposure to an unpredictable food supply increased stress-induced CORT levels but not baseline levels (Buchanan et al., 2003). Last, there are many other factors that can influence the exposure of tissues to CORT, such as the level of corticosteroid binding globulins in the blood and the expression of corticosteroid receptors or enzymes that metabolize CORT in tissues (Schmidt et al., 2008).

CORT levels were manipulated for a relatively long period of time in my study (53 days). However, whereas other methods of hormone manipulation (e.g. silastic implants) constantly elevate hormone levels throughout the treatment period, my method of daily manipulation was transient and CORT levels began to decrease 30 min post-
administration (determined during pilot study; see Materials and methods, Experimental treatments). In addition, in white-crowned sparrows, CORT levels returned to baseline 60 min post-administration using a similar technique (Breuner et al., 1998). Therefore, total exposure to elevated CORT was limited to about 2 h per day in the hand-rearing period and about 1 h per day in the latter part of the treatment period. My method of manipulation would thus be comparable to an individual living in an environment where they are frequently exposed to acute stressors, such as temporary food shortages or frequent encounters with predators. Frequent exposure to acute stressors may become chronically stressful to an individual over time (Clinchy et al., 2004). Indeed, a common paradigm for experiments looking at the physiological effects of chronic stress is to expose individuals to daily acute stressors over several days (e.g. Rich and Romero, 2005).

2.4.2 Developmental stress had sex-specific effects on nestling growth

There were profound sex differences in the effects of developmental stress on nestling growth rates. First, CORT-treated males weighed more than food-restricted and control males throughout the hand-rearing period. This finding is surprising because most studies have found that exposure to elevated glucocorticoid levels during development retards growth (Seckl, 1994; Spencer et al., 2003), although differences in the dose of CORT or method of administration might explain some of the variation between studies. This weight advantage disappeared shortly after nestlings begun feeding independently. Because CORT administration can increase begging rates in nestling birds (Kitaysky et
al., 2001b) and I fed both control and CORT-treated birds to satiation, CORT-treated males may have begged more and been fed more throughout the hand-rearing stage of the experiment. Alternatively, instead of altering behavior and food intake, CORT may have increased anabolic processes. For example, in European starlings (Sturnus vulgaris), CORT treatment in ovo accelerates pectoral muscle development leading to enhanced flight performance (Chin et al., 2009). Glucocorticoids can also increase fat deposition (Asensio et al., 2004). If CORT accelerates growth in male nestlings and increases flight performance, it might decrease the age at which nestlings can fledge. Consistent with this, CORT increases locomotor activity (Breuner et al., 1998) and CORT levels increase prior to fledging or dispersal in many species (e.g. Belthoff and Duffy, 1998; Kern et al., 2001). If nestlings are raised in a poor-quality environment, premature fledging may be beneficial because it would allow a young bird to escape a stressful nest environment, for example if there was intense sibling competition in the nest or an abundance of ectoparasites. Similarly, environmental stressors, including food restriction and pond desiccation, accelerate metamorphosis in spadefoot toads, Scaphiopus hammondii (Denver et al., 1998). In contrast to males, CORT-treated females in the present study weighed less than controls throughout the hand-rearing period. Similarly, early-life glucocorticoid exposure retards growth in zebra finches (Spencer et al., 2003; Spencer and Verhulst, 2007) and humans (Seckl, 1994). Thus, it appears that the effects of glucocorticoids on growth rates are sex- and age-dependent.

Second, there were also sex differences in the effect of food restriction on nestling growth. Food-restricted males weighed the same as control males; however, food-restricted females weighed less than control females. This is in contrast to past studies in
song sparrows (Kempster et al., 2007) and zebra finches (Spencer et al., 2003) in which food restriction decreased growth in both sexes. However, my results are consistent with a study of zebra finches that also found that food restriction decreased growth in females but not males (Martins, 2004). Thus, there may be sex differences in the amount of resources males and females allocate to body growth when exposed to early-life stressors. Males may allocate more resources to body growth at the expense of other systems (e.g. brain, immune system) in order to ensure survival to the fledgling stage. In the following chapters of this thesis I conducted studies to look at the effects of food restriction and CORT treatment on other physiological systems, which will shed light on the different trade-offs and strategies used by males and females when developing in a poor quality environment. Last, because larger nestlings may be fed more by parents and be more likely to fledge (Price and Ydenberg, 1995), the sex-specific effects of food restriction and CORT treatment on nestling growth could provide males with a competitive advantage over their female siblings when raised in a stressful environment (Zanette et al., 2005).

2.4.3 Body size in song sparrows may be a canalized trait

There were no effects of food restriction or CORT treatment on body size at d25, but by d45 CORT-treated birds were smaller than food-restricted and control birds. This was true for both females and males, despite the mass advantage that CORT-treated males exhibited during the hand-rearing period. My PCA for body size included three morphological measures: mass, wing length and tarsus length. Therefore, I interpret these PCA scores as measures of overall body size, but all three measures might not have been
equally affected. CORT-treated birds may be structurally smaller because glucocorticoids can decrease bone formation (Delany et al., 1994). In addition, wing length is related to feather development, and CORT administration impairs feather growth in European starlings (Romero et al., 2005). Despite the effect on body size during the treatment period, there were no effects of either treatment on adult body size. Because my treatments lasted until d60, this suggests that a young song sparrow may compensate for a bad rearing environment by accelerating growth once a stressor subsides even very late during development, well after full adult body size is normally attained. Adult body size may be a canalized trait in song sparrows, showing a large amount of stability even in the face of early-life perturbations [referred to as developmental homeostasis (Mitton and Grant, 1984)]. Therefore, variation in adult body size in song sparrows may be largely determined by variation in genotype with less influence from environmental factors. In support of this, both adult body mass and lean mass of the experimental birds were significantly related to their father’s body mass, and nest identity (natal brood of origin) was significantly related to adult body size. Because I hand-reared nestlings from d3, the relationship between their mass and their father’s mass would be largely due to a common genotype and not a common environment, although I cannot rule out the possibility that the environment before d3 had strong carryover effects on offspring body size. This is in contrast to past studies that have found long-term effects of early-life stress on adult body size (e.g. Searcy et al., 2004). However, my results are consistent with findings from a wild population of song sparrows where morphological measurements of offspring were strongly related to their genetic parents, but not their foster parents (Smith and Dhondt, 1980; also see Merila and Sheldon, 2001).
2.4.4 Developmental stress did not alter body composition

There were no long-term effects of food restriction or CORT treatment on body composition (total body mass, lean mass or fat mass), despite the fact that both treatments altered nestling growth. In contrast, in humans prenatal exposure to famine increases the risk of obesity (Ravelli et al., 1999) and a low birth rate is positively associated with obesity (Rinaudo and Wang, 2012). Catch-up growth may be a particularly important risk factor. For example, rat pups exposed to protein restriction in utero, but then transferred to a high quality diet during the post-partum period, exhibit rapid catch-up growth resulting in a larger body mass and a higher percentage of body fat (Desai et al., 2005). In my study, both food-restricted and CORT-treated females exhibited growth retardation during the hand-rearing period, followed by a period of rapid growth during the latter stage of the treatment period. However, despite experiencing this period of rapid growth, I observed no effect on final body composition. I did observe sex differences in body composition. Males and females had similar total body mass in adulthood, but males had higher lean mass whereas females had higher fat mass.

2.4.5 Developmental stress had sex-specific effects on metabolic rates

The SMRs of birds in the present study were similar to those obtained for house sparrows, *Passer domesticus* (Buchanan et al., 2001), which are similar in size to song sparrows. The average PMR of flying birds is 16 times higher than the BMR (Hinds et al., 1993). Past studies in both red-eyed vireos, *Vireo olivaceous* (Pierce et al., 2005) and house sparrows (Chappell et al., 1999) using similar exercise wheels have obtained PMR
values that were ~10 times higher than BMR. In the present study, PMR values were only ~6 times higher than SMR values. However, the former studies used wild-caught birds, not hand-reared birds, and prolonged periods of captivity can decrease aerobic capacity in birds (Buttemer et al., 2008). Alternatively, the fact that I may have measured SMR and not true BMR could also explain why metabolic scope was lower in the present study.

Both food-restricted and CORT-treated females had higher SMRs than control females. However, SMRs did not differ between males in the three treatment groups. Therefore, developmental stress has sex-specific effects on metabolic rates in song sparrows. Similarly, past studies in birds have found that variation in the rearing environment more strongly affects the metabolic rates of females than males. For example, zebra finch nestlings raised in experimentally enlarged broods have higher SMRs in adulthood, and this effect is stronger in females (Verhulst et al., 2006). In this species, individuals that experience catch-up growth are more likely to experience long-term effects on metabolic rates. For example, nestling zebra finches reared on a low protein diet during the early phase of the nestling period, but then transferred to a high protein diet for the latter part of the nestling period, exhibit catch-up growth and have higher SMRs in adulthood (Criscuolo et al., 2008). In this study, zebra finches reared on a low protein diet throughout the nestling period did not exhibit catch-up growth or an increase in metabolic rates. This suggests that variation in growth patterns during development may contribute to variation in metabolic rates in adulthood. In my study of song sparrows, both food restriction and CORT treatment decreased growth in females, however in adulthood there was no difference in body size or mass between the three treatment groups. Therefore, it is possible that the stress treatments had long-term effects
on the SMRs of females because they altered normal growth patterns of females. In contrast to SMR, there was no effect of either experimental treatment on PMR or metabolic scope. Nest identity was significantly related to both PMR and SMR suggesting that genetic factors also influence variation in metabolic rates in song sparrows. In the present study, time constraints prohibited me from taking more than one measurement of SMR or PMR. However, zebra finches exposed to CORT during development exhibited higher variability in SMR [although only during the treatment period (Spencer and Verhulst, 2008)]. Therefore, it may be of interest in future studies to look at the effects of developmental stress on variability in SMRs or PMRs.

2.4.6 Conclusions

In many species, variation in the early rearing environment can have profound effects on adult phenotype. In particular, exposure to stressors during development can permanently alter physiology and may predispose individuals to disease and negatively affect fitness (McMillen and Robinson, 2005; Monaghan, 2008). In the present study, both food restriction and CORT treatment had long-term effects on SMR in females, but not males, suggesting that the long-term effects of early-life stress on physiology and fitness may be sex-specific. This finding supports past research in zebra finches showing that females are more susceptible to early-life stressors than males (Verhulst et al., 2006; Martin, 2004). In addition, both food restriction and CORT treatment had sex-specific effects on nestling growth rates that exaggerated normal sex differences in nestling mass. This could give males a competitive advantage over their female siblings when being reared in a poor-quality environment (e.g. Zanette et al. 2005). Studies in the following
chapters that look at the effects of developmental stress on other physiological systems elucidate how males and females differentially allocate resources to growth and development when raised in a poor quality environment.
2.5 References


Chapter 3

3 Early-life Stress has Sex-Specific effects on Induced and Constitutive Immune Function in Adult Song Sparrows

3.1 Introduction

The immune system protects organisms from invading pathogens and is essential for survival. Multiple components of the immune system are modulated by environmental conditions, including exposure to stressors (Sapolsky et al., 2000). Long-term exposure to stressors, including food restriction (Martin et al., 2007a), social competition (Hawley et al., 2006), and predator pressure (Boonstra et al., 1998), typically suppresses measures of immune function. In addition, administration of exogenous glucocorticoid hormones, which are produced from the adrenal cortex in response to stress, can also be immunosuppressive (Berger et al., 2005; Martin et al., 2005). Although the traditional view is that stress and glucocorticoid administration suppress the immune system, this is not always the case (reviewed in Sapolsky et al., 2000). For example, in rats, acute stress enhances, while chronic stress suppresses cutaneous immune function (Dhabhar and McEwen, 1997). This short-term enhancement is thought to help mobilize immune cells to sites in the body where they may be needed if wounding occurred (Braude et al., 1999). Therefore, the nature of the effects of stress and glucocorticoid administration on immune function can vary depending on the length of exposure and may depend on other factors including reproductive state (Demas and Nelson, 1998), parasite pressure in the
native environment (Martin et al., 2005), and the specific type of immune activity measured (Stier et al., 2009).

The immunosuppressive effects of chronic stress may help manage the energetic and nutritional costs required to develop, maintain, and use the immune system (Klasing, 2004). The costs of developing the immune system may be particularly relevant for altricial animals, such as songbirds, because they are entirely dependent on their parents for food and thus likely to face limitations in resource availability. In addition, a substantial amount of body growth occurs post-hatch in altricial birds, often at a rapid pace (Ricklefs et al., 1994). For example, in many species of songbirds, nestlings reach their adult body mass within 2-3 weeks of hatching (e.g. European starling, Sturnus vulgaris; Chin et al., 2008). The immune system also undergoes considerable development during this time. For example, nestling tree swallows (Tachycineta bicolor) have lower natural antibody levels and fewer lymphocytes in the blood than adults (Palacios et al., 2009). In addition, the antimicrobial capacity of blood towards the bacterium Escherichia coli increases throughout the nestling period in tree swallows, and is higher in adults than 18-day-old birds, suggesting continued development post-fledging (Stambaugh et al., 2011). In American kestrels (Falco sparverius), antibody production in response to a novel antigen also increases throughout the nestling period (Smits and Bortolotti, 2008). Thus, in altricial nestlings, the development of multiple components of the immune system coincides with the period of rapid postnatal growth, thereby creating high resource demands. As a consequence, a young nestling may face tradeoffs between the development of the immune system and body growth as well as the development of other physiological systems (Lochmiller and Deerenberg, 2000).
Indeed, there is evidence to suggest that resource limitation does affect the immune system during development. For example, fledgling European starlings exhibit suppressed antibody production in response to a novel antigen during exposure to an unpredictable food supply (Buchanan et al., 2003). Similarly, in humans, malnutrition during infancy and childhood has immunosuppressive effects in the short-term (Chandra, 1975; Jones et al., 2010). Exposure to glucocorticoids can also suppress immune function during development (Rubolini et al., 2005; Stier et al., 2009), suggesting that a variety of environmental stressors may modulate immunity early in life. To date, most studies have examined the effects of developmental stress on immune function in the short-term while individuals are still young. There is evidence, however, that early-life stress has long-term, organizational effects on the immune system (Bellinger et al., 2008). Most support for this comes from studies conducted on rodents and humans. For example, exposing male rats to food restriction during the pre and postnatal periods decreased both the number and activity of T lymphocytes at 8 weeks of age (Badr and Mohany, 2011). In humans, prenatal under-nutrition is associated with reduced thymic function (McDade et al., 2001b) and lower antibody production in response to vaccination (McDade et al., 2001a) during adolescence. Very few studies have investigated the long-term effects of early-life stress on immunity in non-mammalian species. In one study, zebra finches reared in experimentally enlarged broods had suppressed innate immunity compared to individuals reared in small broods, when tested under favorable adult conditions (De Coster et al., 2011).

Many studies that determine the effect of stress on immunity use just one or two measures of immune function, especially studies conducted on non-mammalian
vertebrates. However, the immune system is complex and using several measures of immunity may be required to provide a reliable indicator of overall immune function (Keil et al., 2001; Martin et al., 2007b). Specifically, the immune system can be broken down into two mechanistically different branches: 1) the innate (non-specific) immune system, which includes both cellular and humoral components and 2) the acquired (specific) immune system that is comprised of T and B lymphocyte-mediated responses (Janeway, 2005). The long-term effects of early-life stress on immune function may vary depending on whether the innate or acquired immune system is being assessed because the costs to develop and use these two branches of the immune system vary. Specifically, the acquired immune system is more costly to develop, but the innate immune system is more costly to maintain and use (Klasing, 2004). In addition, immune responses can also be categorized as constitutive, which involve the humoral and cellular components of an unchallenged system, or induced, which involve the dynamic components of immunity that are activated in response to an immune challenge (Janeway, 2005). Whether or not early-life stress has different effects on the innate versus the acquired immune system in adulthood, or constitutive versus induced immunity, remains relatively unexplored.

In the current study, I determined the long-term effects of early-life food restriction and CORT treatment on multiple components of the immune system in song sparrows (Melospiza melodia), including measures of both innate and acquired immunity and both constitutive and induced responses (Table 3-1). First, I measured the swelling of the wing-web in response to Phytohemagglutinin (PHA). This represents an induced immune challenge and in house sparrows PHA mobilizes a variety of types of immune cells including lymphocytes, heterophils, monocytes, basophils, and thrombocytes.
Thus this assay is considered a measure of both innate and acquired immunity. Second, I measured leukocyte profiles to quantify the number of lymphocytes, heterophils, and heterophil:lymphocyte (H:L) ratios in the blood. Lastly, I took several measures of constitutive innate immunity (Table 3-1). I took multiple measures of innate immunity because song sparrows are a short-lived species and innate immunity is likely more important for short-lived species than acquired immunity because short-lived species may not live long enough to frequently encounter the same pathogen (Lee, 2006, Martin et al., 2006). In addition, I determined if there were sex differences in the effects of early-life stress on these measures of immunity to see if males and females differ in how they allocate resources to development of the immune system when reared in a stressful environment. Since chronic stress and glucocorticoid exposure typically suppress immune function, I predicted that birds exposed to early-life food restriction or CORT treatment would have weaker immune responses than control subjects. In particular, because development of the acquired immune system overlaps considerably with the period that I administered the experimental treatments, I expected that the measures that assess acquired immunity (swelling response to PHA, circulating lymphocytes) would be most strongly affected.
Table 3-1 Summary of assessment of immune function

<table>
<thead>
<tr>
<th>Assay</th>
<th>Branch</th>
<th>State</th>
<th>Mediated by/Measure of</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swelling response to PHA</td>
<td>Innate and acquired</td>
<td>Induced</td>
<td>Infiltration of several types of immune cells</td>
</tr>
<tr>
<td>Lymphocyte counts</td>
<td>Acquired</td>
<td>Constitutive</td>
<td>Index of circulating lymphocytes</td>
</tr>
<tr>
<td>Heterophil counts</td>
<td>Innate</td>
<td>Constitutive</td>
<td>Index of circulating heterophils</td>
</tr>
<tr>
<td>Microbicidal capacity against E. coli #8739</td>
<td>Innate</td>
<td>Constitutive</td>
<td>Complement system</td>
</tr>
<tr>
<td>Microbicidal capacity against E. coli #51813</td>
<td>Innate</td>
<td>Constitutive</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td>Microbicidal capacity against C. albicans</td>
<td>Innate</td>
<td>Constitutive</td>
<td>Phagocytosis</td>
</tr>
<tr>
<td>Lysis of rabbit erythrocytes</td>
<td>Innate</td>
<td>Constitutive</td>
<td>Complement and natural antibodies</td>
</tr>
<tr>
<td>Agglutination of rabbit erythrocytes</td>
<td>Innate</td>
<td>Constitutive</td>
<td>Natural antibodies</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Innate</td>
<td>Constitutive</td>
<td>Measures lysozyme concentrations</td>
</tr>
</tbody>
</table>

**Note:** “Branch” refers to the arm of the immune system assessed, either innate or acquired. “State” indicates if the response is a static constitutive response or a dynamic induced response.
3.2 Methods

3.2.1 Subjects

The subjects used in the current study were the same as those used in Chapter 2 (Schmidt et al., 2012b) Sample sizes differ slightly due to mortality between the studies. In the current study, a total of 48 birds from 15 broods were used: 17 controls (10 males, 7 females), 16 food-restricted (8 males, 8 females) and 15 CORT-treated (9 females, 6 males). Birds were kept on a long-day photoperiod (16h:8h light:dark) from the time they were brought into captivity until August 16, 2010. At this time, they were switched to a short-day photoperiod (10h:14h light:dark) for the remainder of the experiment. Thus all assessments of immune function occurred when birds were in winter conditions.

3.2.2 Assessment of immune function

I determined the swelling response to an injection of PHA (see below) when birds were ~4.5 months old (129.81 ± 0.31 days), which was ~2.5 months after the end of the stress treatments. I then collected a sterile blood sample (~200 µL) 4 weeks after the PHA injection to prepare thin-film blood smears and perform assays of constitutive innate immune function (see below). Blood samples were collected between 09:00-11:00 AM by brachial venipuncture within 5 min of disturbance because stress can affect the antimicrobial capacity of blood (Millet et al., 2007) as well as heterophil levels (Davis et al., 2008). The vein was cleaned with 70% ethanol to remove hair and dander, allowed to air dry for 20 seconds, cleaned a second time with 70% ethanol, and then allowed to dry completely. The vein was then punctured with a 26-gauge needle and blood was collected
into sterile micro-hematocrit tubes. A small amount of whole blood was used immediately to prepare thin-film blood smears and for the microbicidal assays that used whole blood (*Escherichia coli* #51813 and *Candida albicans*, see below). The remaining blood was centrifuged and plasma was collected and stored at -20°C until processing.

*Phytohemagglutinin skin swelling test*

Injecting PHA *in vivo* and measuring the subsequent skin swelling at the site of injection has been widely used as a measure of an induced immune response in birds, with more swelling taken to indicate a stronger immune response (Lee et al., 2006; Martin et al., 2006; Martin et al., 2005; Rubolini et al., 2005). This procedure is considered a delayed-type hypersensitivity response and in house sparrows results in heightened activity of several types of immune cells including lymphocytes, heterophils, thrombocytes, basophils, and macrophages (Martin et al., 2006). Thus the swelling response to PHA involves both innate and acquired components of the immune system. I injected 20 µL of 5mg/mL PHA-P (Sigma-Aldrich, catalog #L8754, St. Louis, MO, USA) dissolved in sterile Lactated Ringer’s solution into the left wing web. The total amount of PHA injected was thus 0.15 mg, which is comparable to other studies conducted in sparrows (Lee et al., 2006; Martin et al., 2006; Martin et al., 2005). I measured the thickness of the web prior to and 24 h post-injection using a thickness gage (Mitutoyo Corp, ID-C112EBS, series #543, Kawasaki, Japan,). At each time point, I took the average of three separate measurements. The same experimenter performed all injections and swelling measurements between 09:00-11:00 AM and was blind to the treatment group of the birds.
**Leukocyte profiles**

Leukocyte profiles have been widely used as an indicator of physiological condition and immune function across vertebrate taxa (Davis et al., 2008; Harmon, 1998). The concentrations of different types of leukocytes are indicative of different aspects of immune function. Heterophils are phagocytes and form the first line of cellular defense against invading pathogens (Davison et al., 2008). Heterophils rapidly increase in response to inflammation, infection, and stress (Davis et al., 2008; Harmon, 1998). Lymphocytes are involved in both the cell-mediated and humoral components of the acquired immune system (Davison et al., 2008). Having a large number of lymphocytes could indicate a greater capacity to fight invading pathogens and has been found to reliably predict the risk of disease (Davis et al., 2008). Leukocyte profiles were characterized by preparing thin-film blood smears. Smears were allowed to air dry, fixed in 100% methanol, and then stained using a Hemacolor stain set (Harleco, catalog #65044, Gibbstown, NJ, USA). Slides were examined with a light microscope using a 100x oil immersion objective lens. For each slide, I took blood cell counts from 20 digital images of adjacent rectangular fields of view (total number of cells counted = 3630 ± 101.08, approximately 150-200 cells per image). Then, the total number of red blood cells (3621 ± 100.80), lymphocytes (7.96 ±0.67), and heterophils (0.73 ±0.18) were determined in all 20 images. Lymphocyte and heterophil counts were measured as the ratio to red blood cells to control for variation in total cell number. The heterophil to lymphocyte (H:L) ratio was calculated for each slide. H:L ratios increase rapidly in response to infection and exposure to stressors (Davis et al., 2008). However, whether or not this increase is transient or if H:L ratios remain elevated long after exposure to
stressors, is unclear. For one individual, I detected no lymphocytes or heterophils in the 20 images and thus could not calculate the H:L ratio.

*Microbicidal assays*

As part of my assessment of constitutive innate immune function, I measured the antimicrobial capacity of blood or plasma *in vitro*. In order to gain a broad understanding of the ability to limit microbial infection I used three strains of microorganisms following procedures outlined by Millet et al., (2007). First, I determined the ability of previously frozen plasma (frozen for \( \leq 10 \) days) to kill the gram-negative bacterium *E. coli* (American Type Culture Collection [ATCC] #8739; Epower Microorganisms, catalog #0483E7, MicroBiologics, St. Cloud, MN, USA), which is killed primarily by the complement system and can be assessed using plasma as opposed to whole blood (Liebl and Martin, 2009, Millet et al., 2007). I also determined the ability of whole blood to kill a second strain of *E. coli* (ATCC #51813; Epower Microorganisms, catalog #0791E8, MicroBiologics, St. Cloud, MN, USA) as well as one strain of the fungus *C. albicans* (ATCC #10231; Epower Microorganisms, catalog #0443E7, MicroBiologics, St. Cloud, MN, USA). Killing of these latter two strains of microorganisms is primarily accomplished through phagocytosis mediated by cellular components of the blood (Millet et al., 2007). I used a recent modification of the assay, in which bacteria were quantified using spectrophotometry (Liebl and Martin, 2009) that has been used in several species (Cox et al., 2010; Hopkins and Durant, 2011; Liebl and Martin, 2009). I optimized this technique for song sparrows following methods outlined by Liebl and Martin (2009) to determine the optimal incubation times as well as the appropriate blood/plasma and microbe concentrations. The goal of these optimizations was to determine conditions that
allowed blood/plasma to kill ~50% of the microorganism. This was achieved for *C. albicans* (mean killing = 42.73 ± 0.05% across all experimental subjects), however killing of both strains of *E. coli* was low (#8739, mean killing = 7.43 ± 0.01%; #51813, mean killing = 20.26 ± 0.02%) so I used conditions that resulted in the highest antimicrobial activity for these two strains.

For *E. coli* #8739, 36 µL of plasma diluted 1:24 with sterile phosphate-buffered saline (PBS) was added to a sterile micro-centrifuge tube containing 12.5 µL of a working solution of *E. coli* (1x10^5 CFU/mL) and incubated at 37 °C for 45 min. Next, I added 250 µL of sterile tryptic soy broth (TSB) and incubated this mixture for 12 h at 37 °C to allow bacteria to multiply. For *E. coli* #51813, and *C. albicans*, whole blood was added to CO₂-independent cell media (Invitrogen, Carlsbad, CA, USA) immediately after collection. For both of these microorganisms, 36 µL of blood diluted 1:24 with cell media was added to a sterile micro-centrifuge tube containing 12.5 µL of a working solution of the microorganism (1x10^4 CFU/mL for *E. coli* # 51813 and 1x10^5 CFU/mL for *C. albicans*). For *E. coli* #51813, this mixture was allowed to incubate for 2 h at 37 °C and then 250 µL of TSB was added and the solution was incubated for an additional 12 h at 37 °C. For *C. albicans*, the initial blood and fungus mixture was allowed to incubate for 1 h at 30 °C and then 250 µL of TSB was added and the solution was incubated for an additional ~40 h at 30°C. For all three microorganisms, positive controls were processed the same way as samples but contained 36 µL of sterile PBS or cell media without plasma or whole blood. When the second incubation was complete, the concentration of microbes in samples and controls was determined by measuring the absorbance at 300 nm using a nanodrop spectrometer (Liebl and Martin, 2009). I used solutions containing
250 µL sterile TSB plus 48.5 µL sterile PBS (for plasma) or 36 µL of blood diluted 1:24 with cell media plus 12.5 µL sterile PBS (for whole blood) as sterile blanks (Liebl and Martin, 2009). These blanks also served as negative controls for bacterial growth. The anti-microbial capacity of plasma or blood was calculated as \((1 - (\text{absorbance of sample/absorbance of positive control})) \times 100\).

**Hemolysis-hemagglutination assay**

Natural antibody titers and natural antibody-mediated complement activation were quantified using a hemolysis-hemagglutination (HL-HA) assay in which the ability of plasma to agglutinate and lyse rabbit erythrocytes is quantified (Matson et al., 2005). Lysis of rabbit erythrocytes is mediated by interactions between natural antibodies and the complement system, whereas agglutination is dependent on natural antibodies only (Matson et al., 2005). The activity of the complement system and natural antibodies represents one of the first lines of defense of the innate immune system (Janeway, 2005). To quantify lysis and agglutination I followed procedures outlined by Matson et al., (2005). Briefly, I placed 25 µL of previously frozen plasma (frozen for \(\leq 20\) days) into each of the first two columns of a 96-well round-bottom plate. Next, I added 25 µL of Dulbecco’s PBS to columns 2 to 12. The plasma in column 2 was then serially diluted through to column 11 resulting in dilutions from 1 to 1:1024. Column 12 contained only Dulbecco’s PBS and served as a negative control. Chicken plasma was used as a positive control and was added to the first and last rows of each plate. Next, 25 µL of a 1% rabbit red blood cell solution (Hemostat, part #RBA025, Dixon, CA, USA) was added to all wells, at which time plates were covered and allowed to incubate at 37 °C for 90 min.
Following incubation, plates were tilted at a 45° angle for 20 min at room temp. At this time, plates were scanned using a flatbed scanner (Epson Perfection 4990 Photo) at 300 dpi using the positive transparency setting in order to score agglutination. Plates were then allowed to incubate for an additional 70 min and then scanned again to score lysis. Lysis and agglutination were scored as the negative log$_2$ of the last plasma dilution exhibiting each function as in Matson et al. (2005). A single blind observer completed scoring.

**Lysozyme assay**

Lysozyme is an antimicrobial plasma protein and a humoral component of the innate immune system (Janeway, 2005). To quantify plasma lysozyme concentrations, an adaptation of the lysoplate assay (Osserman and Lawlor, 1966) developed by Millet et al. (2007) was used. This assay quantifies bacterial cell lysis through reductions in the opacity of an agar-bacterial suspension. Specifically, lysozyme in the plasma breaks down the peptidoglycans in the cell wall of the gram-negative bacterium *Micrococcus lysodeikticus* resulting in a decrease in the opacity of the suspension. A 50 mg/mL bacterial solution was created by adding 30 mg of lyophilized *M. lysodeikticus* (Sigma-Aldrich, catalog #M3770, St. Louis, MO, USA) to 600 µL of M/15 buffer (pH 6.4). Next, 500 µL of this solution was added to sterile 1% agar (Difco Laboratories, Sparks, MD, USA) in M/15 buffer and kept at 50-60 °C. In each well of a 96-well plate, 150 µL of the agar-bacterial solution was added to 10 µL of plasma sample or standard. The standard curve was created using serial dilutions (10-0.3125 mg/L) of chicken lysozyme (Sigma-
Aldrich, St. Louis, MO, USA). Absorbance was measured at 850 nm using a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA).

### 3.2.3 Data analysis

Statistical analyses were conducted using SPSS version 20 (IBM, Armonk, NY, USA). To analyze swelling of the wing-web in response to PHA, I subtracted the thickness of the wing-web prior to injection from thickness of the wing-web 24 h post-injection to provide an index of the swelling response. I then analyzed these data using a two-way ANOVA with treatment (control, food restriction, CORT) and sex as between-subjects factors. The significant sex x treatment interaction was further analyzed by conducting a one-way ANOVA for each sex with treatment as a between-subjects factor. Significant main effects of treatment were then analyzed using Fisher’s LSD post-hoc test. For all of my statistical analyses (PHA, leukocyte profiles, measures of constitutive innate immunity), I included body mass as a covariate and nest identity (natal nest of origin) as a random factor, however, if these variables were not significant they were removed from the analyses.

To analyze leukocyte profiles, I conducted a two-way ANOVA for each of the 3 measures of leukocyte concentrations (relative lymphocyte number, relative heterophil number, H:L ratio). Sex and treatment were included as between-subjects factors.

In total, I had 6 measures of constitutive innate immune function: 1) microbicidal capacity toward *E. coli* #8739, 2) microbicidal capacity toward *E. coli* #51813, 3) microbicidal capacity toward *C. albicans*, 4) lysis of rabbit erythrocytes, 5) agglutination of rabbit erythrocytes, and 6) plasma lysozyme concentration. Because there is overlap in
the specific components of the innate immune system that these different assays assess, there were many correlations among these different measures. Thus, I first analyzed all 6 measures using a principal component analysis (PCA, see Table 3-2 for factor loadings). Lysozyme concentrations were log_{10} transformed and data from microbicidal assays were square root transformed to reduce positive skew in the data distribution before being entered into the PCA. The PCA revealed 3 components with eigenvalues greater than one that together explained 66.71% of the variance. Microbicidal capacity toward *E. coli* #51813 had a high negative loading onto PC1, while microbicidal capacity toward *C. albicans* had a high positive loading onto PC1, and lysozyme concentrations also had a moderately high positive loading onto PC1. Both microbicidal capacity towards *E. coli* #8739 and lysis of rabbit erythrocytes had high positive loadings onto PC2. Lastly, agglutination of rabbit erythrocytes had a high positive loading onto PC3.

For each principal component (PC), I conducted a two-way ANOVA with sex and treatment as between-subjects factors and PC Score as the dependent variable. Significant treatment x sex interactions were further analyzed by conducting one-way ANOVAs for each sex with treatment as an independent variable. Significant main effects of treatment were analyzed using Fisher’s LSD post-hoc test. In addition, from the PCA it was evident that there was a negative relationship between microbicidal capacity toward *E. coli* #51813 and microbicidal capacity toward *C. albicans*. I used simple linear regressions to determine if this relationship varied among the different treatment groups. All tests were two-tailed and were considered significant at p ≤ 0.05. Data are presented as means ± SEM corrected for the significant covariate or random factor where applicable. Sample
sizes vary for the different analyses because of constraints in the volume of plasma available.

**Table 3-2** Principal component analysis for measures of constitutive innate immune function

<table>
<thead>
<tr>
<th>Factor Loadings</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microbicidal capacity against E.coli #51813</strong></td>
<td>-0.65</td>
<td>-0.02</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>Microbicidal capacity against E.coli #8739</strong></td>
<td>0.26</td>
<td><strong>0.80</strong></td>
<td>0.30</td>
</tr>
<tr>
<td><strong>Microbicidal capacity against C. albicans</strong></td>
<td><strong>0.80</strong></td>
<td>0.08</td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Lysis of rabbit erythrocytes</strong></td>
<td>-0.21</td>
<td><strong>0.78</strong></td>
<td>-0.32</td>
</tr>
<tr>
<td><strong>Agglutination of rabbit erythrocytes</strong></td>
<td>-0.10</td>
<td>0.01</td>
<td><strong>0.91</strong></td>
</tr>
<tr>
<td><strong>Lysozyme Levels</strong></td>
<td><strong>0.50</strong></td>
<td>-0.36</td>
<td>-0.22</td>
</tr>
<tr>
<td><strong>% Variance Explained</strong></td>
<td>23.92</td>
<td>22.99</td>
<td>19.81</td>
</tr>
</tbody>
</table>

Note: PC=principal component. Values in bold loaded highly onto the respective component.
3.3 Results

3.3.1 Swelling Response to PHA

For the swelling response to PHA (Fig 3-1), the treatment x sex interaction was significant ($F_{2,42}=3.39$, $p=0.04$). Neither the main effect of treatment ($F_{2,42}=0.34$, $p=0.72$) nor sex ($F_{1,42}=0.06$, $p=0.80$) were significant. To further analyze the interaction, I conducted one-way ANOVAs for each sex. For males, the main effect of treatment was significant ($F_{2,10}=4.11$, $p=0.05$). Control birds had more swelling in response to PHA than food-restricted ($p=0.03$) or CORT-treated birds ($p=0.04$), which did not differ from each other ($p=0.64$). Nest identity was a nearly significant predictor of swelling in response to PHA in males ($F_{11,10}=2.76$, $p=0.06$). Thus this random factor was left in the analysis. For females, the main effect of treatment was not significant ($F_{2,21}=1.39$, $p=0.27$).

3.3.2 Lymphocyte Counts

For relative lymphocyte levels (Fig 3-2A), neither the treatment x sex interaction ($F_{2,39}=2.39$, $p=0.11$), nor the main effects of treatment ($F_{2,39}=0.16$, $p=0.36$) or sex ($F_{1,39}=3.07$, $p=0.09$) were significant. For relative heterophil levels (Fig 3-2B), the treatment x sex interaction ($F_{2,39}=1.12$, $p=0.34$) was not significant, nor was the main effect of treatment ($F_{2,39}=0.39$, $p=0.68$). The main effect of sex was significant ($F_{1,39}=5.39$, $p=0.03$): males had higher heterophil counts than females (Fig 3-2B). Similarly, for H:L ratios (Fig 3-2C), the treatment x sex interaction was not significant ($F_{2,38}=2.14$, $p=0.13$), nor was the main effect of treatment ($F_{2,38}=1.43$, $p=0.25$). However,
the main effect of sex was significant: (F_{1,38}=7.68, p=0.01): males had higher H:L ratios than females (Fig 3-2C).

Figure 3-1 The long-term effect of early-life food restriction or corticosterone (CORT) treatment on the swelling response to phytohemagglutinin (PHA) in male and female song sparrows. The swelling response was calculated by subtracting the thickness of the wing-web prior to injection from the thickness of the wing web 24 h post-injection. * p≤0.05.
Figure 3-2 The long-term effect of early-life food restriction or corticosterone (CORT) treatment on circulating (A) relative lymphocyte levels (B) relative heterophil levels, and (C) the heterophil:lymphocyte (H:L) ratios in male and female song sparrows. Lymphocyte and heterophil counts were corrected for the number of red blood cells (RBCs) in order to control for variation in the number of cells counted. * p ≤0.05, ** p ≤0.01.
3.3.3  Constitutive Innate Immune Function

For PC1 scores (Fig 3-3A), the treatment x sex interaction was significant ($F_{2,39}=3.82$, $p=0.03$). Neither the main effect of treatment ($F_{2,39}=1.12$, $p=0.34$) nor sex ($F_{1,39}=0.000$, $p=0.99$) were significant. To further analyze the interaction, I conducted one-way ANOVAs for each sex. For males, the main effect of treatment was significant ($F_{2,19}=4.06$, $p=0.03$). CORT-treated males had higher PC1 scores than control ($p=0.03$) and food-restricted ($p=0.02$) males. PC1 scores did not differ between control and food-restricted males ($p=0.76$). For females, the main effect of treatment was not significant ($F_{2,20}=0.53$, $p=0.60$). For PC2 scores (Fig 3-3B), neither the treatment x sex interaction ($F_{2,39}=0.003$, $p=0.997$) nor the main effects of treatment ($F_{2,39}=0.58$, $p=0.57$) or sex ($F_{1,39}=0.42$, $p=0.52$) were significant. For PC3 scores (Fig 3-3C), neither the treatment x sex interaction ($F_{2,39}=0.44$, $p=0.65$) nor the main effect of treatment ($F_{2,39}=0.73$, $p=0.49$) were significant. The main effect of sex was significant ($F_{1,39}=6.84$, $p=0.01$): females had higher PC3 scores than males (Fig 3-3C).

It was evident from the PCA on the measures of constitutive innate immune function that microbicidal capacity towards *E. coli* #51813 was negatively correlated with microbicidal capacity towards *C. albicans* (Table 1). This was surprising since killing of both these strains of microbes is primarily accomplished through phagocytosis mediated by cellular components of the blood. To determine if this relationship varied for the different treatment groups, I used simple linear regressions. For control subjects (Fig 3-4A), microbicidal capacity towards *E. coli* #51813 was not significantly related to microbicidal capacity towards *C. albicans* ($r^2=0.03$, $p=0.52$). For food-restricted subjects
Figure 3-3 The long-term effect of early-life food restriction or corticosterone (CORT) treatment on (A) principal component (PC) 1 scores, (B) PC2 scores, and (C) PC3 scores for the measures of constitutive innate immune function in male and female song sparrows. The measures of constitutive innate immune function included in the PCA and their respective loadings onto each PC are outlined in Table 2. * p≤0.05, ** p≤0.01.
(Fig 3-4B), there was a significant negative relationship between the microbicidal capacity towards \textit{E. coli} #51813 and the microbicidal capacity towards \textit{C. albicans} \((r^2=0.26, p=0.04)\). For CORT-treated subjects (Fig 3-4C), there was a trend for a negative relationship between the microbicidal capacity towards \textit{E. coli} #51813 and the microbicidal capacity towards \textit{C. albicans} but this relationship was not significant \((r^2=0.19, p=0.10)\).
Figure 3-4 Simple linear regressions showing the relationship between microbicidal capacity against *E. coli* #51813 and microbicidal capacity against *C. albicans* for song sparrows raised under three conditions: (A) control, (B) food restriction, or (C) corticosterone (CORT) treatment.
3.4 Discussion

I found that early-life food restriction and CORT treatment had sex-specific effects on both innate and acquired immunity in adult song sparrows. First, as predicted, both food-restricted and CORT-treated males exhibited less swelling of the wing-web in response to PHA. However, neither stressor affected the swelling response in females. Second, males exposed to CORT-treatment during development had higher PC1 scores for the measures of constitutive innate immunity than control or food-restricted males. This indicates that CORT-treated males had lower microbicidal activity toward *E. coli* #51813, but higher microbicidal activity towards *C. albicans*, than control or food-restricted males. Again, there was no effect of either stressor on PC scores for constitutive innate immunity in females. There was no effect of early-life food restriction or CORT treatment on leukocyte profiles in males or females, but males had higher relative heterophil counts and H:L ratios than females. These results suggest that male and female song sparrows may differ in how they allocate resources to development of the immune system when reared in stressful or food-limited conditions.

3.4.1 Swelling response to PHA

Both early-life food restriction and CORT treatment decreased the swelling response to PHA in adult males, but not females. PHA stimulates the proliferation of T lymphocytes and in house sparrows induces the rapid infiltration of several types of immune cells to the site of injection (Martin et al., 2006). These cells are involved in both the innate (e.g. heterophils) and acquired (lymphocytes) branches of the immune system. Although several studies have shown that stress or CORT treatment affect the immediate
swelling response to PHA (Berger et al., 2005; Martin et al., 2005; Rubolini et al., 2005), to my knowledge this is the first study to show that chronic CORT and food-restriction suppress swelling long after (~2.5 months) the stressors are terminated. This suggests that early-life stress has long-lasting programming effects on the development of induced immunity in male song sparrows. Similarly, male rats exposed to food restriction during the pre- and postnatal periods exhibited reductions in T lymphocyte activity at 8 weeks of age (Badr and Mohany, 2011). Humans exposed to prenatal under-nutrition produced fewer antibodies in response to a vaccine in adolescence (McDade et al., 2001a), again suggesting that variation in the early environment can have long-term effects on induced immunity.

Interestingly, in the present study, the effects of early-life food restriction and CORT treatment on induced immunity were observed in males but not females. Previous studies have also found that variation in the early rearing environment has sex-specific effects on immunity. For example, in a year of low resource availability, skin swelling in response to PHA was suppressed by increased sibling competition in nestling male European starlings, but was unaffected in females (Chin et al., 2005). Sex differences in the effects of early-life stress may reflect differences in how males and females allocate resources to the development of physiological traits when reared in a poor quality environment. In Chapter 2, I found that female song sparrows exposed to food-restriction or CORT treatment weighed less than control females during the hand-rearing period (Schmidt et al., 2012b). In contrast, food-restricted males weighed the same as controls and CORT-treated males actually weighed more than control males during the hand-rearing period (Chapter 2; Schmidt et al., 2012b). These results, combined with the
results of the present chapter, suggest that in response to early-life stress males may allocate more resources to body growth at the expense of immune system development, perhaps as a strategy to increase short-term survival. In contrast, female song sparrows appear to invest more in development of the immune system at the expense of body growth, which may help ensure survival over a longer timescale.

3.4.2 Leukocyte profiles

There were no long-term effects of food restriction or CORT treatment on heterophil counts or H:L ratios. However males had higher relative heterophil counts and H:L ratios than females. It is well known that stress and glucocorticoids increase heterophil levels and H:L ratios during, or shortly after, exposure to stressors in birds (Davis et al., 2008). It is possible that the effects of stress on H:L ratios are transient and may subside once the stressor is terminated in song sparrows. In the current study, relative heterophil levels (mean=0.73 ±0.18) and H:L ratios were very low compared to what I have previously observed for free-living song sparrows (mean=2.90 ± 0.56; Schmidt et al., 2012a). Since heterophils and H:L ratios increase in response to infection (Davis et al., 2008; Harmon, 1998) this could be explained by the fact that my birds were reared in captivity and therefore likely would have been free of infection. In addition, since subjects were habituated to captivity they were all living in a relatively low stress environment at the time blood samples were taken, which may further contribute to the low heterophil counts and H:L ratios.

I found no long-term effect of early-life food restriction or CORT treatment on relative lymphocyte counts. Several previous studies in birds have shown that stress
decreases lymphocyte levels (Cirule et al., 2012; Ruiz et al., 2002). However, these studies examined the effects of stress on lymphocytes during or shortly after exposure to the stressor. Therefore, it is possible that food-restricted and CORT-treated subjects in my study had lower lymphocyte counts during the treatment period but that this effect disappeared once the stressors were terminated. However, previous studies have found long-term effects of early-life stress on circulating lymphocyte levels in mammals. For example, adult male rats born to females that were subjected to chronic restraint stress during pregnancy had fewer lymphocytes in the blood and higher eosinophil and neutrophil (mammalian equivalent of heterophils) counts (Llorente et al., 2002). In addition, rats exposed to food restriction in the pre and postnatal periods also had lower levels of T lymphocytes in the blood at 8 weeks of age (Badr and Mohany, 2011).

Lymphocytes mediate the actions of both the cell-mediated and humoral components of the acquired immune system. Since I measured lymphocytes in unchallenged individuals, this provides a measure of constitutive acquired immunity. My only measure of induced immunity was the response to PHA, which reflects both innate and acquired immune function. Comparing antibody production to a previously encountered antigen would provide valuable information on the long-term effect of early-life stress on immunological memory, a unique component of the acquired immune system.

3.4.3 Constitutive innate immune function

PC1 scores for the measures of constitutive innate immunity were higher in CORT-treated males than control and food-restricted males. There was no effect of early-life food restriction or CORT treatment on PC1 scores in females. Microbicidal activity
against *E. coli* #51813 had a high negative loading onto PC1, whereas microbicidal activity against *C. albicans* had a high positive loading onto PC1 (Table 3-2). This indicates that males exposed to chronic CORT treatment during development have a decreased ability to eliminate at least one strain of *E. coli*. However, there was no effect of early-life stress on PC2 scores, which were largely explained by variation in microbicidal capacity against *E. coli* #8739 and lysis of rabbit erythrocytes. Therefore, stress did not affect the ability to eliminate both strains of *E. coli*. This could be explained by the fact that these two strains of *E. coli* are eliminated by different components of the innate immune system. Elimination of *E. coli* #51813 is achieved through phagocytosis mediated by cellular components of the blood whereas elimination of *E. coli* #8739 is primarily mediated by the complement system (Liebl and Martin, 2009; Millet et al., 2007). Therefore, early-life CORT treatment appears to affect some components of the innate immune system in song sparrows, such as the activity of phagocytes, but not others, such as the complement system. In contrast, adult zebra finches reared in experimentally enlarged broods exhibited decreased lysis of rabbit erythrocytes when housed in favorable adult conditions (De Coster et al., 2011). The fact that lysis of rabbit erythrocytes is primarily mediated by complement proteins (Millet et al., 2007) suggests that variation in the early rearing environment did have long-term effects on the complement system. Therefore, the effects of early-life stress on specific components of innate immunity may be species-specific and also may depend on the type of stressor. Interestingly, in the same study in zebra finches there was no effect of natal brood size on innate immunity when birds were housed in unfavorable adult conditions (De Coster et
al., 2011), suggesting that the long-term effects of early-life stress may also vary depending on the quality of the adult environment.

As mentioned above, higher PC1 scores in CORT-treated males also indicates that CORT-treated males were better able to eliminate C. albicans than control or food-restricted males. Thus, early-life CORT treatment may have long-lasting enhancing effects on some measures of constitutive innate immunity. This is surprising because most studies have found that chronic stress and glucocorticoid treatment are immunosuppressive (Dhabhar, 2009). However, immunosupression is not always the case. For example, rats exposed to chronic prenatal stress produced more antibodies in response to a novel antigen at 65 days of age than rats not exposed to prenatal stress (Klein and Rager, 1995). Thus, it appears that even chronic stress can sometimes enhance components of immune function and that the effects of stress and glucocorticoids on immunity are complex and likely depend on many variables (Dhabhar, 2009).

Another surprising finding in the current study was that microbicidal capacity against E. coli #51813 and C. albicans were negatively correlated (r=-0.39, p=0.01 for all subjects). Elimination of both these two strains of microbes is primarily achieved through phagocytosis mediated by cells in the blood, particularly macrophages and heterophils (Millet et al., 2007). Since their elimination depends on the same components of constitutive innate immunity, I expected them to be positively correlated. However, cells of the innate immune system recognize pathogens via pathogen recognition receptors (Akira et al., 2006) and distinct receptors may recognize these two microorganisms since they represent different kingdoms from two distinct domains of life (bacteria versus fungus). Therefore, the ability to eliminate one strain of microorganism does not
necessarily reflect the ability to eliminate all strains of microorganisms demonstrating the importance of including multiple strains of microbes from different kingdoms in studies that are designed to assess innate immune function. My findings suggest that there may be tradeoffs in the ability to eliminate different kinds of microbes. This is further supported by the fact that the negative relationship between antimicrobial activity against E. coli #51813 versus C. albicans was stronger for individuals exposed to early-life stress than control subjects. Indeed this relationship was significant for food-restricted subjects, but not controls. Thus the ability to invest in multiple pathogen recognition receptors that are capable of recognizing many different types of microbes may be further limited in individuals raised in a stressful environment.

3.4.4 Sex differences in immune function

Females had higher PC3 scores than males. PC3 was largely explained by variation in the agglutination of rabbit erythrocytes, which is mediated by natural antibodies (Matson et al., 2005). This suggests that females have higher natural antibody titers than males. As mentioned above, I also detected sex differences in leukocyte profiles. Males had higher relative heterophil counts and H:L ratios than females. Heterophils are phagocytes and are a component of cellular innate immunity. In contrast, natural antibodies are a component of the humoral innate immune system. This suggests that males may rely more on at least some cellular components of the innate immune system, whereas females may rely more on some humoral components of innate immunity. This sex difference in antibody titers is consistent with several previous studies showing that females have higher levels of circulating antibodies and are capable
of mounting stronger primary and secondary antibody responses to antigenic challenges (reviewed in Klein, 2000).

3.4.5 Conclusions

My results demonstrate that there are profound sex differences in the long-term effects of early-life food restriction and CORT treatment on immunity in song sparrows. Males exposed to early-life stress mounted weaker induced immune responses to PHA, showed impaired ability of peripheral blood to eliminate one strain of bacteria, and enhanced ability of peripheral blood to eliminate a strain of fungus. In contrast, females exposed to early-life food restriction or CORT treatment exhibited no alterations in either induced or constitutive immunity. I suggest that in response to developmental stress males may tradeoff development of the immune system in favor of body growth in order to increase the chance of short-term survival. However, females appear to allocate more resources to development of the immune system even when exposed to a stressful rearing environment, which may increase the chance of long-term survival. In addition, not only may individuals face tradeoffs in the development of distinct physiological systems, but they may also face tradeoffs in their ability to invest in different components of immune function (Lee, 2006). Specifically, investing in defenses to eliminate one kind of microorganism may come at the cost of investing in defenses to eliminate another kind of microorganism and early-life stress appears to intensify these tradeoffs. Importantly, my results suggest that early-life food restriction and CORT treatment do not suppress all aspects of immune function and that they may even enhance some components. This
illustrates the importance of comprehensive assessments of immune function when investigating the effects of stress or glucocorticoid exposure on immunity.
3.5 References


Chapter 4

4 Developmental Programming of the HPA and HPG Axes by Early-life Stress in Male and Female Song Sparrows

4.1 Introduction

Variation in the early rearing environment can have long-term organizational effects on many physiological and neural systems (McMillen and Robinson, 2005; Welberg and Seckl, 2001). This process is referred to as developmental programming and commonly results from exposure to stressors, such as nutritional restriction or other factors that elevate glucocorticoid levels (Welberg and Seckl, 2001). In particular, exposure to early-life stressors has long-term effects on endocrine regulation. For example, prenatal nutritional restriction affects development of the hypothalamic-pituitary-adrenal (HPA) axis in rats, resulting in exaggerated increases in corticosterone (CORT) in response to acute stressors in adulthood (Vieau et al., 2007) and impairments in negative feedback (Navarrete et al., 2007). Early-life stressors can also affect development of the hypothalamic-pituitary-gonadal (HPG) axis in mammals, which may negatively affect adult sexual behaviour and reproduction (Rhind et al., 2001; Ward and Weisz, 1980; Guzmán et al., 2006).

Less is known about how early-life stress affects the HPA and HPG axes in non-mammalian species, but since these endocrine systems are evolutionarily homologous
across vertebrates it is possible that similar effects occur in other taxa. In support of this, adult western scrub jays (*Aphelocoma californica*) exposed to food restriction in the nestling period exhibit larger increases in CORT in response to acute stress when compared to control animals (Pravosudov and Kitaysky, 2006). Juvenile frogs (*Xenopus laevis*) treated with CORT as tadpoles have higher baseline CORT levels and fewer glucocorticoid receptors in the brain (Hu et al., 2008). To date, most studies in non-mammalian species have focused on baseline and stress-induced CORT levels, and it is unclear if early-life stress affects other components of HPA axis regulation (e.g. negative feedback), or if early-life stress affects regulation of the HPG axis.

Understanding the effects of early-life stress on endocrine regulation is important because variation in the regulation of the HPA and HPG axes is related to health and fitness in many species. For example, experimentally elevating glucocorticoid levels decreases parental care and induces nest abandonment in pied flycatchers (*Ficedula hypoleuca*; Silverin, 1986). In addition, the magnitude of the stress response is negatively associated with survival in European white storks (*Ciconia ciconia*; Blas et al., 2007) and in song sparrows in some years (*Melospiza melodia*; MacDougall-Shackleton et al., 2009, 2013). In marine iguanas (*Amblyrhynchus cristatus*), the ability of the synthetic glucocorticoid dexamethasone (DEX) to suppress CORT (a widely used index of negative feedback; Watson et al., 2006) is associated with increased survival during an El Niño event (Romero and Wikelski, 2010). HPA axis regulation is also related to measures of phenotypic condition and quality. For example, in free-living song sparrows, the magnitude of the stress response is inversely related to song complexity (MacDougall-Shackleton et al., 2009; Schmidt et al., 2012a). In the same species, the
ability of DEX to suppress endogenous CORT levels is related to body size and circulating heterophil levels (Schmidt et al., 2012a).

Variation in sex steroid levels and HPG axis regulation is also related to health and fitness. For example, testosterone can increase inter-male aggression in some species of songbirds (Wingfield et al., 1990; Wingfield, 1984). Although this may be beneficial early in the breeding season when males are actively establishing territories and acquiring mates, chronic exposure to elevated testosterone levels can have negative physiological effects, including suppression of the immune system (Owen-Ashley et al., 2004), increased metabolic rates (Wikelski et al., 1999), and decreased body mass and fat stores (Ketterson et al., 1991). In addition, experimentally elevating testosterone levels in male songbirds can reduce paternal care and decrease the number of offspring surviving per brood (Hegner and Wingfield, 1987; Silverin, 1980). In female songbirds, ovarian sex steroids, particularly 17β-estradiol (hereafter estradiol), are involved in reproduction and thus important to reproductive success. In support of this, estradiol levels are elevated during ovulation and oviposition in female song sparrows (Wingfield, 1984) and estradiol treatment increases the production of yolk precursors in female European starlings (Sturnus vulgaris; Christians and Williams, 1999). In addition, estradiol stimulates reproductive behaviour in female songbirds including copulation displays and chitter vocalizations (Searcy and Capp, 1997; Searcy and Marler, 1981).

In the current study, I determined the effects of early-life stress (food restriction or CORT treatment) on regulation of the HPA and HPG axes in song sparrows. For the HPA axis, I used a protocol that allowed me to assess 3 components of HPA axis regulation (Schmidt et al., 2012a): 1) the response of CORT to acute restraint, 2) efficacy
of negative feedback by determining the ability of DEX to suppress endogenous CORT levels, and 3) adrenal sensitivity to exogenous adrenocorticotropic hormone (ACTH). Variation in stress-induced CORT levels can be due to variation in release of corticotropin-releasing hormone (CRH), ACTH, or CORT, as well as to variation in the degree to which individuals perceive the stressor as a threat. In contrast, injecting ACTH isolates variation in the stress response that is specifically due to variation in the sensitivity of the adrenal cortex to ACTH. To characterize HPG axis regulation, I measured initial (unchallenged) sex steroid levels (testosterone in males and estradiol in females) as well as sex steroid levels after a gonadotropin-releasing hormone (GnRH) challenge. Testosterone levels increase rapidly in response to GnRH in male songbirds (Jawor et al., 2006) and this may provide an indication of the testosterone levels that would be observed after a social challenge from a male conspecific or after an interaction with a female (Moore, 1983; Wingfield, 1985). To my knowledge, this is the first study to measure plasma estradiol levels in a female songbird after exposure to exogenous GnRH, although a previous study showed that plasma luteinizing hormone (LH) does increase in response to a GnRH challenge in female white-browed sparrow weavers (Plocepasser mahali [Wingfield et al., 1991]). My hypothesis was that both HPA and HPG axis regulation would be affected by early-life food restriction and CORT exposure. Specifically, I predicted that individuals exposed to early-life stress would have larger increases in CORT in response to restraint and ACTH, smaller decreases in CORT in response to DEX, and lower levels of circulating sex steroids before and after a GnRH challenge.
4.2 Methods

4.2.1 Subjects

The birds used in this study were the same as those used in Chapter 2 (Schmidt et al., 2012b). However, sample sizes differed slightly due to mortality of some subjects: control n=9 males, n=7 females, food restriction n=8 males, n=7 females, and CORT treatment n=6 males, n=8 females.

4.2.2 Characterization of the HPA axis

When birds were initially brought into captivity, they were housed on a long day photoperiod (16L:8D) until 16 August 2010, at which time they were switched to short days (10L:14D) to simulate winter conditions. Birds were transferred back to long days (photostimulated) 4 weeks before characterization of the HPA axis. Females were ~8 months old (mean=242.32, SEM=0.99 days) at the time that I characterized HPA axis regulation (Fig 4-1) and males were ~11.5 months old (mean=350.91, SEM=2.11 days). Females were sampled at a younger age than males because females were required for another experiment measuring the effects of early-life stress on the behavioural response of females to male song, which took place after this study (Schmidt et al. 2013a). However, in songbirds, the HPA axis is fully matured by 3 weeks of age (Wada et al., 2007; Wada et al., 2009) so this difference in age should have little effect on my measures of HPA axis regulation.
**Figure 4-1** Experimental timeline used to determine the long-term effects of early-life stress on activity of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes in song sparrows. d= day of age.

*Blood sampling and injections*

I characterized HPA axis regulation following a protocol that has been previously used in free-living song sparrows (Schmidt et al., 2012a) and chukar (*Alectoris chukar*; Dickens et al., 2009). This protocol allowed us to characterize three components of HPA axis regulation: 1) the response to a standardized restraint stressor, 2) negative feedback of the HPA axis, and 3) adrenal sensitivity to ACTH. Blood samples were collected by brachial venipuncture with a 26-gauge needle and collection into heparinized micro-hematocrit tubes. First, I took an initial baseline blood sample within 3 min of disturbance (entering the room) between 9:30-11:30 AM. Birds were then placed in a cloth bag for 30 min, at which time I took a second blood sample to calculate the response of CORT to an acute stressor (calculated as 30 min CORT levels – 3 min CORT levels). Immediately following this, birds received an injection of DEX (1 mg/kg, product no. 2301, Sandoz Canada, Inc., Boucherville, QC, CA) into the pectoralis muscle. Birds were then placed back in the cloth bag for an additional 60 min, at which time I took a third blood sample to determine the ability of DEX to suppress CORT (calculated as 90 min CORT levels – 30 min CORT levels). Immediately following this, birds received an injection of porcine ACTH (25 IU/kg, product A6303, Sigma Aldrich, St. Louis, MO,
U.S.A.) into the pectoralis muscle. Birds were then placed back in their home cage for 30 min, at which time I took a fourth and final blood sample to determine the response of CORT to ACTH (calculated as 120 min CORT levels – 90 min CORT levels). I collected no more than a total of 200 µL of blood from each bird. Blood was centrifuged at 13,000 g for 10 min and plasma was then collected and stored at -20°C until processing.

CORT assay

CORT was quantified in unextracted plasma using a commercially available 125I radioimmunoassay (07-120103, MP Biomedicals, Santa Ana, CA, U.S.A.) that has been previously validated for use in song sparrows (Newman et al., 2008b). Samples were analyzed randomly with respect to treatment and sex in four assays. Plasma was diluted 1:50 with steroid diluent (provided with the kit) and samples were analyzed in duplicate by adding 50 µL of the diluted plasma sample to each tube. The intra-assay coefficient of variation was 11.53% for a low control (12.5 pg/tube) and 4.78% for a high control (125 pg/tube). The inter-assay coefficient of variation was 11.52% for the low control and 4.73% for the high control. CORT concentrations in all samples fell within the range of the standard curve (1.56 – 250 pg/tube). The CORT antibody used in the RIA has a low cross reactivity to DEX (<0.01%) according to the manufacturer.

4.2.3  GnRH challenge

Blood sampling and Injections

I characterized the HPG axis using GnRH challenges one week after characterization of HPA axis regulation (Fig 4- 1). Blood samples were collected and
processed as described above. To determine the appropriate dose of GnRH to inject, I conducted a pilot study using adult male song sparrows that were caught in the spring of 2010 (Fig. 4-2). Birds were brought into captivity and housed on a long day photoperiod in individual cages. The pilot study was conducted one month after birds had been in captivity. I took an initial blood sample within 3 min of disturbance. Subjects then received an injection of either a low (0.05 µg/g, n=4) or a high (0.10 µg/g, n=4) dose of chicken GnRH-I (54-8-23, The American Peptide Company, Inc., Sunnyvale, CA, U.S.A.) into the pectoralis muscle. Additional blood samples were collected 30, 60 and 120 min post-injection. I collected no more than a total of 200 µL of blood from each bird. Similar doses and time points were used in a study of dark-eyed juncos (Junco hyemalis; Jawor et al., 2006). Testosterone levels peaked 30 min after injection and decreased to near baseline levels by 60 min (see Results Section). Peak testosterone levels were similar for the low and the high dose. Thus, I used the low dose of GnRH for the experiment and collected blood samples prior to and 30 and 60 min post-injection. Although I did not conduct pilot studies in female song sparrows, I chose the same dose and time points to use in females. Initial blood samples were collected between 10:00 AM - 12:00 PM. Samples collected 30 min post-injection represent maximum testosterone levels (Jawor et al., 2006). Since levels return to near baseline by 60 min post-injection, this time-point is indicative of the efficacy of negative feedback of the HPG axis.
Figure 4-2 Pilot study conducted in adult male song sparrows to determine the appropriate dose of gonadotropin-releasing hormone (GnRH) to use for GnRH challenges. Low dose = 0.05 µg/g, high dose = 0.10 µg/g. Points not sharing a letter are significantly different for p<0.05.

Testosterone assay

Testosterone was quantified in unextracted plasma using a commercially available enzyme immunoassay (1-2403, Salimetrics, State College, PA, U.S.A.) in males only. The instructions provided by the manufacturer were followed exactly. This kit has previously been used to measure testosterone in songbird plasma (Washburn et al., 2007), but has not specifically been used in song sparrows. In order to validate the assay for use with song sparrows, song sparrow plasma was serially diluted and compared to the standard curve (Chard, 1995) using an ANCOVA. A lack of significant interaction between the serial dilution and the standard curve indicates that the line slopes are similar (Newman et al., 2008a). The interaction term was not significant (F_{1,8} = 0.306, p = 0.595).
indicating that the assay is effective for measuring testosterone in plasma from song sparrows.

For samples, plasma collected prior to injection of GnRH was diluted 1:28 with assay buffer (provided with the kit) and plasma collected 30 and 60 min after GnRH injection was diluted 1:50. Samples were analyzed in duplicate by adding 25 µL of diluted plasma to each well. Samples were analyzed randomly with respect to treatment in 3 assays. The intra-assay coefficient of variation was 5.81% for a low control (1 pg/well) and 2.20% for a high control (6 pg/well). Testosterone levels in all samples fell within the range of the standard curve (0.15 – 15 pg/well).

*Estradiol assay*

Plasma estradiol levels were quantified in females only by extracting steroids from plasma using solid phase extraction with C18 columns (Bond Elut LRC-C18 OH, 500 mg sorbent, Agilent Technologies, Mississauga, ON, CA) as described previously (Brummelte et al., 2010; Charlier et al., 2011; Taves et al., 2011). Briefly, columns were primed with 3 mL HPLC-grade methanol and equilibrated with 10 mL deionized water. Next, 10 mL of deionized water was added to plasma (17 µL) and samples were then loaded onto columns. Columns were then washed with 10 mL 40% HPLC-grade methanol. Finally, steroids were eluted with 5 mL 90% HPLC-grade methanol. Eluates were dried using a vacuum concentrator (SpeedVac, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 40 °C. Samples were resuspended in 340 µL phosphate-buffered saline with 0.1% gelatin (PBSg) containing 0.8% absolute ethanol. I calculated recovery by adding 3 pg of radioinert estradiol to plasma before extraction and comparing estradiol
levels in spiked (n=2) versus unspiked (n=2) plasma. Recovery of estradiol in plasma was 88%.

Estradiol was measured using a commercially available double antibody $^{125}$I radioimmunoassay (DSL-4800; Beckman Coulter Canada, Inc., Mississauga, ON, Canada) that has previously been used in song sparrows (Heimovics et al., 2012) and white-crowned sparrows (Charlier et al., 2011). The assay was modified following procedures outlined by Charlier et al. (2010) to increase assay sensitivity. Samples were run in singleton (as in Charlier et al., 2011) because estradiol levels were low and only a small volume of plasma was available. All samples were analyzed in one assay using 300 µL of the resuspended sample (equivalent to 15 µL of plasma/tube). The intra-assay coefficient of variation for a control (1.28 pg/tube) was 2.06%. Estradiol levels in all samples fell within the range of the standard curve (0.2 - 20 pg/tube).

4.2.4 Data analysis

Statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). When necessary, data were transformed ($\log_{10}$) to reduce positive skew. To determine the effects of the treatments on HPA axis regulation, I conducted ANOVAs with treatment and sex as between-subject factors and each measure of HPA regulation (response to restraint, negative feedback, adrenal sensitivity to ACTH) as the dependent variables. Significant main effects of treatment were analyzed using LSD post-hoc tests. For all analyses of endocrine regulation, I included body mass as a covariate and nest identity (natal nest of origin) as a random factor. If the covariate or random factor were not significant, they were removed from the analyses.
For the GnRH pilot study, I compared testosterone levels using a repeated measures ANOVA with time (0, 30, 60, 120 min) as a within-subject factor and dose (low or high) as a between-subjects factor. Significant main effects of time were analyzed using LSD post-hoc tests. To determine the effect of the treatments on testosterone levels in males, I conducted ANOVAs with treatment as a between-subject factor and testosterone levels at each time point (0, 30 or 60 min) as the dependent variable. GnRH had no effect on plasma estradiol levels in females (see Results section). Therefore, for each subject, I calculated the average concentration of estradiol across the three time-points and compared the effect of treatment on average estradiol levels using an ANOVA with treatment as a between-subject factor. Significant main effects of treatment were analyzed using LSD post-hoc tests. All tests were two tailed and were considered significant for \( p \leq 0.05 \). Data represent mean ± SEM corrected for the significant random factor where applicable.

4.3 Results

4.3.1 HPA axis

I used ANOVAs to determine the effects of treatment and sex on the three measures of HPA regulation. For the response of CORT to restraint stress (Fig 4-3A), neither the treatment x sex interaction (\( F_{2,38}=0.26, p=0.77 \)), nor the main effects of treatment (\( F_{2,38}=1.57, p=0.22 \)) or sex (\( F_{1,38}=0.91, p=0.35 \)) were significant. Similarly, for the response of CORT to DEX challenge (Fig 4-3B), neither the treatment x sex
Figure 4-3 The long-term effects of early-life food restriction and corticosterone (CORT) treatment on (A) the acute stress response (CORT levels post-restraint [30 min] – baseline [3 min]), (B) negative feedback of the HPA axis (CORT levels post-DEX [90 min] – post-restraint [30 min]), and (C) adrenal sensitivity to ACTH (CORT levels post-ACTH [120 min] – CORT levels post-DEX [90 min]) in adult song sparrows. ACTH = adrenocorticotropic hormone, Dex = dexamethasone. *p<0.05, **p<0.01.
interaction ($F_{2,39}=0.46$, $p=0.64$), nor the main effects of either treatment ($F_{2,39}=1.21$, $p=0.31$) or sex ($F_{1,39}=0.43$, $p=0.51$) were significant. For the response of CORT to ACTH challenge (Fig 4-3C), the treatment x sex interaction ($F_{2,39}=1.33$, $p=0.28$) and the main effect of sex ($F_{1,39}=0.51$, $p=0.48$) were not significant. However the main effect of treatment was significant ($F_{2,39}=4.34$, $p=0.02$). ACTH challenge induced larger increases in CORT in CORT-treated birds than control ($p=0.009$) or food-restricted ($p=0.02$) birds. ACTH challenge increased CORT to similar levels in control and food-restricted subjects ($p=0.75$).

4.3.2 HPG axis

_Pilot Study_

For the pilot study (Fig 4-2), neither the time x dose interaction ($F_{3,18}=0.14$, $p=0.94$) nor the main effect of dose ($F_{1,6}=0.04$, $p=0.86$) were significant. The main effect of time was significant ($F_{3,18}=15.754$, $p<0.001$). Testosterone levels prior to injection of GnRH were significantly lower than testosterone levels 30 min post-injection ($p=0.003$), but were not significantly different from testosterone levels 60 ($p=0.29$) or 120 ($p=0.12$) min post-injection. Testosterone levels 30 min post-injection of GnRH were significantly higher than testosterone levels 60 ($p=0.009$) and 120 ($p<0.001$) min post-injection. Testosterone levels 60 min post-injection of GnRH were significantly higher than testosterone levels 120 min post-injection ($p=0.01$). Thus, for both doses of GnRH, testosterone levels peaked 30 min post-injection and had decreased to near pre-injection levels by 60 min.
**Males**

For initial testosterone levels, (Fig 4-4A), the main effect of treatment was significant ($F_{2.20}=2.60$, $p=0.046$). Control males had significantly lower testosterone levels than CORT-treated males ($p=0.02$). Initial testosterone levels did not differ between control and food-restricted males ($p=0.17$) or food-restricted and CORT-treated males ($p=0.20$). For testosterone levels 30 min post-injection of GnRH (Fig 4-4B), the main effect of treatment was not significant ($F_{2.9}=0.30$, $p=0.75$). Nest identity was significantly related to peak testosterone levels ($F_{11,9}=3.79$, $p=0.03$). Lastly, for testosterone levels 60 min post-injection of GnRH (Fig 4-4C), the main effect of treatment was not significant ($F_{2.20}=0.23$, $p=0.80$).

**Females**

There was no significant difference in estradiol levels collected at the three time-points (0 min = 104.76 ± 12.68 pg/mL, 30 min = 169.88 ± 43.83 pg/mL, 60 min = 113.03 ± 14.72 pg/mL; $F_{2.38}=1.50$, $p=0.24$), indicating that GnRH did not increase plasma estradiol levels in females. Therefore, I calculated the average estradiol concentration across the three-time points for each subject (Fig 4-5). The main effect of treatment was significant ($F_{2.19}=4.43$, $p=0.03$). Average plasma estradiol levels were higher in control females than food-restricted ($p=0.01$) or CORT-treated ($p=0.02$) females. Estradiol levels did not differ between food-restricted and CORT-treated females ($p=0.76$).
Figure 4.4 The long-term effects of early-life food restriction and corticosterone (CORT) treatment on (A) initial testosterone levels, (B) testosterone levels 30 min following an injection of exogenous gonadotropin-releasing hormone (GnRH), and (C) testosterone levels 60 min following an injection of exogenous GnRH in adult male song sparrows. *p<0.05.
The long-term effects of early-life food restriction and corticosterone (CORT) treatment on plasma 17β-estradiol levels in adult female song sparrows. Plasma was collected prior to and 30 and 60 min following an injection of exogenous gonadotropin-releasing hormone (GnRH). GnRH had no effect on plasma estradiol levels so estradiol levels were averaged across the three time-points for each subject. *p<0.05, **p<0.01.

4.4 Discussion

Early-life food restriction and CORT treatment had long-term effects on the production and regulation of glucocorticoids and sex steroids in male and female song sparrows. First, as predicted, both male and female CORT-treated birds exhibited exaggerated increases in CORT in response to exogenous ACTH. This suggests that exposure to elevated CORT early in development permanently affects CORT synthesis by the adrenal cortex. However, contrary to my initial prediction, neither stressor affected the response of CORT to restraint stress or efficacy of negative feedback. Second,
surprisingly, males treated with CORT during development had higher initial testosterone levels than control males. However, neither CORT treatment nor food restriction affected the response of testosterone to a GnRH challenge. Lastly, as predicted, females exposed to either early-life stressor had lower plasma estradiol levels than control females. Therefore, interestingly, early-life CORT treatment had opposite effects on plasma sex steroid levels in male and female song sparrows.

4.4.1 HPA axis

Individuals exposed to early-life CORT treatment had an exaggerated CORT response to ACTH challenge in adulthood, when compared to control and food-restricted birds. Injecting ACTH specifically isolates variation in CORT synthesis that is due to sensitivity of the adrenal cortex to ACTH. Thus, my results suggest that early-life CORT treatment induces permanent physiological changes in the adrenal cortex of song sparrows that result in exaggerated CORT synthesis long after the treatment subsides. Variation in adrenal CORT synthesis in response to ACTH could be due to many factors. For example, the adrenals of CORT-treated birds may have more ACTH receptors or increased expression of the steroidogenic enzymes that are involved in CORT synthesis. In support of this, exposure to prenatal stress increases expression of the ACTH receptor and steroidogenic acute regulatory protein (StAR) in the adrenal gland of fetal sheep (Ovis aries; Edwards et al., 2002). I did not find an effect of early food restriction on the CORT response to ACTH. In contrast, a recent study found that food restriction throughout the nestling and juvenile period resulted in reduced response to ACTH challenge in zebra finches (Taeniopygia guttata; Kriengwatana et al., in review). Thus,
potential effects of food restriction on adrenal sensitivity to ACTH may depend on the timing and duration of the manipulation.

There was no effect of food restriction or CORT treatment on the response of CORT to restraint stress or negative feedback of the HPA axis (as assessed by the ability of DEX to suppress CORT). This is in contrast to several studies in mammals that have shown that a variety of prenatal and postnatal stressors permanently alter HPA axis regulation, leading to exaggerated increases in CORT in response to acute stress and impaired negative feedback (Matthews, 2002). For example, adult rats separated from their mother for a few hours per day in the first 2 weeks of life have a heightened stress response, impaired negative feedback, and reduced expression of the glucocorticoid receptor in the brain (Kalinichev et al., 2002; Ladd et al., 2004). Similarly, exposing western scrub jays to food restriction early in life leads to exaggerated increases in CORT in response to restraint in adulthood (Pravosudov and Kitaysky, 2006). Nestling zebra finches treated with CORT also show enhanced CORT synthesis in response to a restraint stressor in adulthood (Spencer et al., 2009). In my study, the increase in CORT in response to restraint was low, especially when compared to free-living song sparrows (Newman et al., 2008b; Schmidt et al., 2012a), which may make it difficult to detect an effect of early life stress on the acute stress response. If I had used a different test stressor that was more effective at increasing CORT levels (e.g. exposure to a predator; Pakkala et al., 2013) it is possible that I would have observed an effect of early-life stress on the acute stress response or negative feedback.
4.4.2 HPG axis

**Males**

Initial testosterone levels were higher in CORT-treated males than in food-restricted or control males. However, there was no effect of either treatment on testosterone levels after a GnRH challenge. Increased initial testosterone levels could be due to increased hypothalamic GnRH secretion, pituitary gonadotropin secretion, or testicular testosterone synthesis. For example, in male sheep, prenatal undernutrition increased steroidogenic enzyme expression in the fetal testis (Rae et al., 2002b) and follicle-stimulating hormone secretion by the adult pituitary (Rae et al., 2002a). In contrast to my findings, numerous studies in mammals have found that exposure to elevated glucocorticoid levels or stressors decreases testosterone levels by inducing apoptosis of Leydig cells and inhibiting the activity of testicular steroidogenic enzymes (reviewed in Hardy et al., 2005). However, most studies have examined the immediate effects of stress or glucocorticoid exposure, as opposed to the long-term effects. To my knowledge, this is the first study to determine the long-term effects of early-life stress on the HPG axis in an avian species. Previous studies in adult songbirds found that chronic CORT treatment had minimal effect on circulating testosterone levels in song sparrows (Wingfield and Silverin, 1986) or tree sparrows (*Passer montanus*; Astheimer et al., 2000). Therefore, the effect of chronic CORT treatment on the HPG axis in male songbirds may be exclusive to exposure during development, perhaps because the HPG axis is still developing during this time.
Testosterone regulates aggressive behaviour in breeding song sparrows (Wingfield and Wada, 1989; Wingfield and Hahn, 1994; Wingfield, 1985). Therefore, CORT-treated males may behave more aggressively towards conspecifics during the breeding season, which may facilitate the defense of mates or food resources. A male who experienced a stressful upbringing may be less attractive to females, for example because males exposed to early-life stress sing less complex song (Chapter 5; Schmidt et al., 2013b). Therefore, increased aggression and mate guarding could help a less attractive male secure a mate and avoid losing paternity to a more attractive male.

However, this may come at a cost because chronic exposure to elevated testosterone levels can have negative physiological effects including suppression of the immune system (Owen-Ashley et al., 2004), increased metabolic rates (Wikelski et al., 1999), and decreased body condition (Ketterson et al., 1991). Thus, one hypothesis is that exposure to elevated CORT during development programs males to pursue a fast pace of life and to increase testosterone levels in order to maximize reproductive success in the short-term at the expense of long-term survival.

**Females**

Plasma estradiol levels did not increase in response to exogenous GnRH in females. To my knowledge, this is the first study to measure plasma estradiol levels in a female songbird following a GnRH challenge. However, past studies have found that exogenous GnRH also fails to increase plasma testosterone levels in female songbirds (Spinney et al., 2006; Wingfield et al., 1991). Testosterone did increase in response to exogenous GnRH in female dark-eyed juncos, but only during the period of egg development when females have the greatest number of hierarchical follicles (Jawor et
al., 2007), which are the follicles that are most active in producing steroids (Johnson, 2000). Therefore, it is possible that in female song sparrows circulating estradiol also only increases in response to exogenous GnRH during egg development. None of the females in my study laid eggs, suggesting that they did not reach this stage of ovarian development. Thus, it is possible that in the current study gonadal estradiol synthesis was constrained by the stage of ovarian development.

In contrast to males, exposure to either food restriction or CORT treatment early in life resulted in a 50% reduction in circulating estradiol levels in females. This suggests that early-life stress has pronounced organizational effects on regulation of the HPG axis in female song sparrows. Similarly, exposure to early-life stress has long-term effects on regulation of the HPG axis and reproduction in female mammals. For example, female rats exposed to prenatal or postnatal protein restriction had lower estradiol levels compared to control subjects at 21 days of age. The protein-restricted females also exhibited a decline in fertility rates in adulthood compared to controls (Guzmán et al., 2006). In addition, in sheep, feeding pregnant ewes a high nutrient intake reduces placental mass and results in fetal growth restriction (Da Silva et al., 2002). Female fetuses born to over-nourished ewes have fewer ovarian follicles (Da Silva et al., 2002) and lower plasma progesterone levels (Da Silva et al., 2003). I did not examine ovaries in the current study, but it is possible that circulating estradiol levels were lower in females exposed to early-life stress because these individuals had fewer or smaller ovarian follicles.

Estradiol is important for reproduction in female songbirds. Circulating estradiol levels peak during ovulation and oviposition in song sparrows (Wingfield, 1984).
Estradiol stimulates the production of the yolk precursors vitellogenin and very-low density lipoprotein by the liver (Wallace, 1985) and albumen by the oviduct (Brant and Nalbandov, 1956), all of which are essential for egg production. Estradiol also stimulates female reproductive behaviour, including copulation solicitation displays and chitter vocalizations (Searcy and Capp, 1997; Searcy and Marler, 1981). Interestingly, in Chapter 6, I found that females exposed to food restriction or CORT treatment during development were less selective in their behavioural response to conspecific versus heterospecific (white-crowned sparrow; *Zonotrichia leucophrys*) songs (Schmidt et al., 2013a). This difference in behaviour was paralleled by differences in the neural induction of the immediate early gene *ZENK* in auditory forebrain regions (Chapter 6; Schmidt et al., 2013a). In that study, females were implanted with estradiol so it is unlikely that circulating estradiol levels varied between treatment groups at the time of testing. However, it is possible that variation in estradiol levels during development or in adulthood prior to receiving the implants had long-term effects on areas of the brain that regulate female reproductive behaviour.

4.4.3 Conclusions

Exposure to early-life food restriction or CORT treatment had pronounced effects on endocrine regulation in adult song sparrows. Exposure to CORT-treatment during development lead to exaggerated increases in CORT in response to a standardized dose of ACTH. Therefore, exposure to increased CORT levels during development may permanently affect adrenal CORT synthesis in song sparrows. Males treated with CORT during development also had higher initial testosterone levels than control males. This
could potentially affect aggressive behaviour towards conspecifics, physiological condition, or paternal care and reproductive success. Lastly, exposure to either stressor during development decreased plasma estradiol levels in females, which may affect egg production as well as reproductive behavior and subsequent interactions with male conspecifics. This suggests that early-life CORT exposure may have opposite effects on male and female songbirds, with regards to plasma sex steroid levels. These results illustrate the importance of developmental conditions in inter-individual variation in endocrine regulation.
4.5 References


Chapter 5

5 Early-Life Stress affects Song Complexity, Song Learning, and Volume of the Brain Nucleus RA in Adult Male Song Sparrows

5.1 Introduction

In many species of songbirds, the species-specific characteristics of song are acquired early in life by learning from adult tutors. Male songbirds typically sing for two primary reasons: to defend territories and to attract females (Catchpole and Slater 1995). Therefore, variation in a male’s ability to learn song could have important fitness consequences. When selecting a mate, female songbirds may attend to a variety of features of song (Nowicki and Searcy 2004). For example, females may prefer males that sing more complex song (MacDougall-Shackleton 1997), which is typically defined as the number of song types or syllables in their repertoire. In addition, females may attend to features of song that reflect the quality of song learning, for example preferring song types characteristic of the natal population (MacDougall-Shackleton et al., 2001) or songs that contain fewer invented notes (Nowicki et al., 2002a). Another feature of song that may be important for attracting females is vocal performance, for example, the ability of a male to produce physically challenging notes such as trills that require rapid and precise coordination of vocal tract movement (Podos, 1997; Ballentine et al., 2004).
The learning and production of song are controlled by a series of inter-connected brain nuclei that are collectively referred to as the song-control system. The nuclei that make up the song-control system exhibit rapid growth during the period of song acquisition (Bottjer et al., 1985). Variation in the size of the song-control nuclei in adulthood may be related to variation in the quality of song production. For example, the volume of HVC may be positively correlated with song complexity between species (DeVoogd et al., 1993) and the volume of both HVC and the robust nucleus of the arcopallium (RA) may be positively correlated with song complexity within species (Garamszegi and Eens 2004). However, this relationship may not hold true for all species. For example the size of HVC is not related to song complexity in red-winged blackbirds (*Agelaius phoeniceus* [Kirn et al., 1989]). In addition to song complexity, the volume of the song-control nuclei may be related to other song features such as the length of song phrases (Airey and DeVoogd 2000) or song bouts (Bernard et al., 1996) and the stereotypy of note structure (Smith et al., 1997a).

As mentioned above, female songbirds may attend to features of song when selecting a mate. Females may benefit by attending to particular song attributes when they accurately reflect the phenotypic quality of potential mates. For example, results from field studies suggest that song complexity may indicate a male’s physiological condition (Pfaff et al., 2007), ability to successfully defend a territory (Hiebert et al., 1989), or future parental effort (Buchanan and Catchpole 2000). The Developmental Stress Hypothesis, originally called the Nutritional Stress Hypothesis, posits that learned song may honestly advertise the phenotypic quality of the singer, because song learning and the development of the song-control system occur early in life when young birds are
exposed to a variety of environmental stressors, such as food restriction (Nowicki et al., 1998; Nowicki et al., 2002b). One prediction derived from this hypothesis is that males who are exposed to more developmental stressors should show poor development of the song-control system and as a result should produce a low-quality song in adulthood. In addition to the song-control system and song production, early-life stress may have widespread effects on the development of other physiological and neural systems (Buchanan et al., 2003; Verhulst et al., 2006; Spencer et al., 2009; Farrell et al., 2011). Therefore males exposed to early-life stress may both produce low-quality song and be of poorer phenotypic quality in general. Furthermore, some males may be more resistant to stressors such that song could indicate both the quality of a male’s early-rearing environment and/or his genotype.

In recent years, evidence for the Developmental Stress Hypothesis has accumulated (reviewed in MacDougall-Shackleton and Spencer 2012). For example, exposing nestling zebra finches (Taeniopygia guttata) to food restriction or treatment with the glucocorticoid hormone corticosterone (CORT) reduces the growth of HVC (Buchanan et al., 2004) and results in shorter songs with fewer syllables (Spencer et al., 2003). However, another study of zebra finches found no effect of food restriction on syllable repertoire size, although food restriction decreased the accuracy of song learning (Brumm et al., 2009). Similarly, swamp sparrows (Melospiza georgiana) exposed to food restriction early in life show reduced song-learning accuracy and smaller volumes of RA relative to the size of the telencephalon (Nowicki et al., 2002b). Song-type repertoire size was not affected in this study, but swamp sparrows have small song repertoires (two to four song types) with little variation among males in a population, which could make it
difficult to detect an effect of stress on this aspect of song (Nowicki et al., 2002b). Indeed, most studies examining the effects of stress on song complexity to date have focused on species with relatively small song-type or syllable repertoire sizes such as zebra finches, Bengalese finches (*Lonchura striata domestica*), and swamp sparrows. In contrast, European starlings (*Sturnus vulgaris*), which have large song-type repertoires, sing fewer song types in adulthood after exposure to an unpredictable food supply in the fledgling period (Spencer et al., 2004). In addition, most studies have focused on measures of song complexity and song-learning accuracy and little is known about how early-life stress affects measures of vocal performance.

In the current study, I examined the effect of early-life food restriction or CORT treatment on song sparrows (*Melospiza melodia*). Song sparrows are closed-ended song learners meaning that they do not alter their song repertoires in adulthood (Nordby et al., 2002). Song sparrows have complex repertoires that typically consist of 5-12 song types composed of 20-50 unique syllables (Pfaff et al., 2007; Stewart and MacDougall-Shackleton 2008). In addition, there is some evidence that song complexity is a sexually selected trait in this species that may influence female mating preferences and/or male-male competition (Hiebert et al., 1989; Reid et al., 2004; Searcy 1984; although see Beecher et al., 2000). My first objective was to determine whether early-life stress affects several song features including song complexity (number of song types and syllables), song-learning accuracy, vocal performance, and song-type stereotypy. I was particularly interested in a measure of vocal performance not previously tested, trill deviation, which is the speed of frequency modulation within a trill, thought to be constrained by a tradeoff between trill rate and frequency bandwidth (Podos 1997). The second objective was to
determine whether early-life stress affects the song control system including the volumes of HVC, RA, and area X, as well as the number of neurons in HVC. HVC, RA, and area X are important for the learning and production of song. I chose to measure the number of neurons in HVC because CORT affects the total number of neurons as well as the number of proliferating cells in this region in adult song sparrows (Newman et al., 2010). Lastly, I also recorded the song repertoires of the fathers of the experimental subjects to determine whether paternal song quality is related to the offspring’s adult song quality following exposure to developmental stress. If a particular song feature is resistant to the effects of early-life stress and has a significant relationship with paternal song production (despite the fact that nestlings were not reared by their parents), then this feature may be more strongly influenced by hereditary factors than environmental factors. If so, then certain song features may indicate a male’s genetic quality and ability to resist stressors as opposed to the amount of stress experienced during development. Consistent with previous studies, I predicted that early-life stress would affect features of song that are linked to sensory learning during song acquisition (song complexity, song-learning accuracy), as well as the song-control system. However, because development of vocal performance is believed to occur later in ontogeny during the sensorimotor phase of song learning (Podos et al., 2009), which in song sparrows can occur throughout the first year of life and long after the stress treatments were terminated, early-life stress might not affect vocal performance in this species.
5.2 Methods

5.2.1 Subjects

I located song sparrow nests near Newboro, Ontario (44°38’N, 76°20’W) in May and June 2010 (Schmidt et al., 2012). I recorded the complete song repertoire of the resident male associated with each nest (see below for details on song recording) in order to quantify paternal song complexity and trill deviation for each experimental subject. Since extra-pair paternity is low in this population (consistently <10% of nestlings [Potvin and MacDougall-Shackleton 2009]) the resident male was presumed to be the genetic father of nestlings collected from each nest. Male subjects used in the current study were the same as the male subjects used in Chapter 2 (control: n=9, food-restricted: n=8, CORT-treated: n=6).

5.2.2 Song tutoring

Male song sparrows learn their songs from adult tutors during a sensitive period in development (Marler and Peters 1987). Song acquisition occurs primarily during 10-50 days post-hatch but can extend up to 200 days. However, 90% of songs are learnt before 90 days post-hatch (Marler and Peters 1987). Initially, all subjects were housed in one holding room. Males were exposed to recorded song sparrow song beginning at day 10-25 (mean = 16.91, SD= 4.64) and ending at day 116-131 (mean=122.91, SD= 4.64). All subjects were exposed to recorded song sparrow song for exactly 106 days (total of 258 min/day, see below). Details on the song stimuli used in these recordings are given below. Because there is evidence that song learning can be enhanced by exposure to live
tutors (Beecher and Burt 2004), I caught four adult males near the area where nests were located and housed these males in the room with the experimental subjects. These live tutors thus provided song models to supplement playback of recorded song. Live tutors were housed individually in cages placed at opposite corners of the room. The location of their cages was changed every 2 weeks so that all subjects had equal exposure to the four live tutors. One live tutor sang frequently, whereas the other three were never heard singing.

When subjects were 60 days old I moved them to individual cages inside sound attenuation chambers (model AB-2000 S, Eckel Noise Control Technologies, Cambridge, MA, U.S.A.). This was done because birds began producing subsong around this age and I wanted to ensure that the tutor song was the primary acoustic stimulus birds were exposed to. Because I had eight available chambers, each chamber housed three subjects (one from each of the three treatment groups). Subjects were housed on different shelves within the chamber and thus were in auditory, but not visual, contact with one another. Siblings were not housed in the same chamber. The recorded tutor songs were played at constant amplitude through a speaker (Optimus Pro-X44AV) that was placed out of the birds’ field of view. When birds were ~150 days old (about 5 months) they were returned to the holding room, still housed in individual cages (see Fig 5-1 for timeline). By this age, a young song sparrow has primarily finished acquiring tutor song (Marler and Peters 1987), although late song learning may occur with exposure to live, singing tutors (Nordby et al. 2001).

Song tutoring stimuli were prepared using Syrinx software (v. 2.6h; J Burt; www.syrinxpc.com). Tutor playbacks consisted of 32 unique song types from four
individual male song sparrows (see Fig 5-2 for example). I recorded these males in the spring of 2010 from the same population as the experimental subjects. I did not use songs from any of the experimental subjects’ fathers. I chose song recordings of high quality (based on signal-to-noise ratio and absence of background sound). Each song type was filtered to remove background noise and then peak amplitude was equalized. This number of tutor singers and exemplars was chosen as representative of the number of singing individuals that a young song sparrow is likely to be exposed to in the wild.

Songs were played at a rate of six songs per minute, which mimics natural song rate. I created two playback sequences that contained the 32 song types played in random order, each one repeated eight times before switching to the next song type (total of 256 songs over 43 min). Each playback was played once in the morning, afternoon, and evening with 1 h of silence between the two playbacks. In total, birds were exposed to 258 min of song per day.

![Figure 5-1 Experimental timeline](image)
Figure 5-2 Example spectrograms of three song types recorded from male song sparrows that were used to tutor the experimental subjects during the song-learning period.
5.2.3 Song recording

When subjects were initially brought in to captivity they were kept on a long day photoperiod (16 h light: 8 h dark [16L:8D]) until 16 August 2010. At this time they were switched to short days (10L:14D) in order to simulate winter conditions. This was necessary to break photorefractoriness and bring subjects into a state of photosensitivity so that I could later induce a photostimulated reproductive state at the start of song recording. Birds were transferred back to long days (16L:8D) five weeks before their song was recorded. For song recording, subjects were moved to outdoor aviaries on the roof of the Advanced Facility for Avian Research at the University of Western Ontario from May to July 2011 when they were ~1 year old. Subjects were recorded outdoors, instead of inside in sound-attenuation chambers, because many individuals did not sing frequently indoors. I recorded each subject’s song repertoire using Marantz Professional Solid State PMD 671 recorders and Telinga Twin Science parabolic microphones (Uppsala, Sweden). I considered a repertoire to be recorded in full after either 300 consecutive or 450 nonconsecutive songs had been recorded (Pfaff et al. 2007). One bird in the food-restricted group only sang sporadically and I was unable to record a sufficient number of songs from this individual; therefore, I excluded this male from all behavioural analyses. I also recorded the one live adult male tutor that sang frequently during the song-tutoring period at this time to quantify song-learning accuracy (see below).

The territorial male where each nest was located (the presumed father) was recorded in the spring of 2010 at the field site using the equipment described above. Males had been previously captured using mistnets and each had a unique combination of
colour bands to aid in identification. I was unable to record the father for one male in the control group because this nest was found the day nestlings hatched and the resident male sang too infrequently at that time.

5.2.4 Song analyses

The experimenter performing the song analyses was blind to the treatment condition of the birds for all attributes of song that were studied.

Song complexity

In my study population, free-living individuals typically sing 5-12 unique song types (Pfaff et al. 2007) composed of 20-50 syllables (Stewart and MacDougall-Shackleton 2008). For both the experimental subjects and their fathers, I created spectrograms of songs using Syrinx software and identified song types and syllables visually. I determined the total number of song types in a bird’s repertoire to determine song-type repertoire size (as in Pfaff et al. 2007) and the total number of syllables to determine syllable repertoire size (as in Stewart and MacDougall-Shackleton 2008). Song sparrows sing with eventual variety, meaning that they sing one song type several times before switching to a new song type. I identified each unique song type as the birds switched to a new song in their repertoires. Slight variations of a given song type (e.g. the addition or omission of a syllable) were not counted as new song types. I then identified each unique syllable in all song types to quantify syllable repertoire size.
**Song-learning accuracy**

I used Raven Pro sound analysis software (v. 1.4, Cornell Lab of Ornithology, Ithaca, NY, U.S.A) to quantify the accuracy with which subjects copied the tutor songs. The primary tutor stimuli that the subject birds had been exposed to were the recorded songs. When exposed to recorded songs, as opposed to live male tutors, song sparrows tend to copy only elements of a tutor song and not the entire song. Therefore, I looked for similarity between all of the syllables in a subject bird’s repertoire and all of the tutor syllables they were exposed to during development (total of 200 syllables) as opposed to similarity between whole song types. This included all syllables from the 32 unique song types that comprised the tutor playback sequences, as well as the syllables from the live male tutor that was observed singing frequently. First, I conducted spectrogram cross-correlations (SPCCs) of all the syllables in a male’s repertoire against all of the tutor syllables to identify the tutor syllable with the highest SPCC. This tutor syllable was assumed to be the syllable that the bird was attempting to imitate. I visually confirmed that the subject’s and the tutor’s syllables were highly similar in cases where the program gave a high SPCC value and were less similar in cases where the program gave a low SPCC value (See Fig 5-3). To obtain a more accurate estimate of copy accuracy, I next calculated the average pairwise SPCC value for 10 renditions of each copied syllable against the tutor syllable that served as the model (as in Nowicki et al. 2002b). Lastly, I averaged the SPCCs across all syllables for a given bird to produce one overall learning accuracy score for each subject. This score was then used in statistical analyses.
Figure 5-3 Examples of 9 spectrogram cross correlations (SPCC) showing the similarity between the tutor syllable (on the left) and the tutee’s rendition of the syllable (on the right). The numeric values represent the SPCC scores.

Trill deviation

I analyzed trill deviation for the experimental subjects and their fathers using Raven Pro Sound Analysis Software following methods outlined by Podos (1997, 2001). Song sparrows, which produce frequency-modulated trill syllables, show a characteristic trade-off in performance between the number of syllables that can be produced per unit time (trill rate), and the frequency that these syllables can encompass (frequency
This tradeoff served as the basis for my analysis of trill deviation. The trill rate for each trill type was calculated from oscillograms by dividing the number of notes produced by the duration of each trill. Spectrograms were used to measure frequency bandwidth, which was calculated as the difference between the maximum and minimum frequencies of each trill at 90% signal energy. This 90% criterion was chosen to eliminate effects of background noise and to control for varying signal strength between recordings. I determined the average trill rate and frequency bandwidth for 10 renditions of each trill type.

Frequency bandwidth was plotted as a function of trill rate following Podos (1997). Briefly, a regression line was calculated by binning trills by trill rate in 5 Hz increments, and selecting the trill with the highest frequency bandwidth from each bin (six bins, from 0 – 30 Hz). A linear regression was then calculated on these select data points. The resulting regression line represents an upper bound for performance (Blackburn et al. 1992) and showed a triangular distribution with a negative slope (R=-98.94, p=0.001). Finally, deviation from the upper performance limit (distance from the regression line) was calculated as the minimum orthogonal distance from each of the individual trill types measured to the upper-bound regression line. Therefore, this deviation represents a relative measure of vocal performance, with small deviations indicating higher-performing trills. I averaged the deviation scores across all trill types in a bird’s repertoire in order to produce one overall trill deviation score per subject. This average score was then used in statistical analyses with a lower score (lower deviation from performance limit) indicating higher vocal performance. In addition to the average
deviation, I also analyzed the minimum deviation to characterize maximal performance for each bird.

**Song-type stereotypy**

Although different song types are easily distinguishable from one another, song sparrows will often produce slight variations of a given song type. In previous studies, variation in the consistency with which a male sings has been assumed to be indicative of vocal performance with the assumption that the highest-quality singers perform with the highest level of consistency (reviewed in Podos et al. 2009). To determine the variability with which a bird sang each of their songs, I used Raven Pro Sound Analysis Software to calculate the average pair-wise SPCC between 10 renditions of each song type that made up a bird’s repertoire. I then averaged the SPCC values across all song types to produce one stereotypy score for each subject. In addition to the average SPCC values, I also analyzed the maximum SPCC value to characterize maximal performance for each bird.

**5.2.5 Immunocytochemistry**

Subjects were euthanized using an overdose of isoflurane once their song repertoires had been completely recorded. Brains were immediately extracted and the two hemispheres were bisected using a scalpel blade. One hemisphere was immediately frozen on dry ice to be used in another study. The remaining hemisphere was post-fixed in 5% acrolein for 1.5 h. Within a treatment group, I used the left hemisphere for half of the subjects and the right hemisphere for the remaining half of the subjects. Brains were then rinsed three times in 0.1 M phosphate buffered saline (PBS) for 30 min and then
transferred to 30% sucrose for cryoprotection. Once brains had sunk they were rapidly
frozen on powdered dry ice and stored at -80°C until processing.

Brains were sectioned in the sagittal plane at 40 μm thickness using a cryostat.
Every second section was collected and processed for NeuN immunoreactivity. NeuN is a
protein expressed in the majority of mature neuronal cell types (Mullen et al. 1992) and
was used to calculate the volumes of the song control nuclei HVC, RA, and area X, as
well as to count the number of NeuN+ cells in HVC to provide an estimate of the number
of neurons in this region (see below). In each run (six runs total) I processed four brains
for NeuN immunocytochemistry (ICC) with at least one bird from each treatment group.
First, free-floating sections were washed twice in 0.1 M PBS, followed by incubation in
0.5% hydrogen peroxide (H₂O₂) for 15 min. Next, sections were washed three times in
PBS followed by incubation in 10% normal goat serum (Vector, Burlingame, CA,
U.S.A.) diluted in 0.3% Triton in PBS (PBST) for 1 h. Sections were then incubated for
20 h in a primary monoclonal antibody (raised in mouse, MAB377, Millipore,
Billerica, MA, U.S.A.) diluted 1:2000 in 0.3% PBST (Philmore et al. 2006; Newman et al. 2010).
Sections were then washed 3x in 0.1% PBST and incubated for 1 h in biotinylated
secondary antibody (goat-anti mouse IgG, Vector, Burlingame, CA) diluted 1:250 in
0.3% PBST. Next, sections were washed three times in 0.1% PBST, followed by
incubation in avidin-biotin horseradish-peroxidase complex (Vector Elite ABC kit) for 1
h. Sections were then washed twice in 0.1% PBST and immediately visualized by
exposing them to diaminobenzidine solution (Sigma Fast-DAB) and washed three times
in PBS. Lastly, sections were mounted onto gelatin-coated slides, dehydrated with a
series of graded ethanol, cleared in solvent (Harleco Neo-Clear, EMD Chemicals,

5.2.6 Microscopy

Slides were coded so that the experimenter making measurements was blind to the treatment group. I calculated the volume of the song nuclei HVC, RA, and area X using a Leica Digital CCD camera mounted on a Leica DM5000B light microscope (Leica Microsystems Inc., Richmond Hill, ON, Canada). I captured images from every NeuN-labelled section that contained the song nuclei of interest and then used Leica Application suite software to trace the area of these regions of interests. I combined the area measurements using the formula for the volume of a frustrum to calculate the volume of HVC, RA and area X in one hemisphere. I multiplied all measurements by two to estimate the total volume of the nuclei in both hemispheres. Song nuclei volumes do not differ between the two hemispheres (Tramonti et al. 2000). To obtain an index of total brain size, slides were scanned at 2400 dpi into a computer using a flatbed scanner with a transparency adapter. Using imageJ64 software (National Institutes of Health, Bethesda, MD, U.S.A.), I measured the area of the total telencephalon for 11 sections centered on the largest cross section of area X. Area measurements were combined using the formula for the volume of a frustrum to produce a measure of midtelencephalon volume (as in MacDougall-Shackleton et al. 1998).

In addition to measuring the volume of HVC, I also estimated the number of neurons (as NeuN+ cells) in HVC following previously published protocols (Newman et al. 2010; Hall et al. 2012). To estimate the number of neurons in HVC, I used the optical
fractionator method (Glasser et al. 2007). NeuN+ cells were counted in every sixth section (240 μm intervals) that contained HVC. Using a x20 objective lens, I used the Leica Application suite software driving a motorized stage to cover HVC with a 270 μm x 270 μm sampling grid. I next moved the section to the first square in the sampling grid using a x63 oil objective. At each point in the sampling grid the counting frame was 30 μm x 30 μm. I measured the thickness of each tissue section by focusing on the top and bottom edges in each counting frame (average section thickness after processing = 9.70 ± 0.16 μm). I did not count cells in the top or bottom 1 μm (guard zones). I focused throughout the Z-axis in 1 μm increments and counted all of the neurons that came into focus within 7 μm (optical dissector height) from the top guard zone. The total number of NeuN+ cells was then estimated using the following equation: total number of NeuN+ cells = n x (1/ssf) x (1/asf) x (1/hsf), where n is the total number of cells counted, ssf is the section sampling frame (1/6, since I counted every sixth section), asf is the area sectioning frame (30 μm²/270 μm²), and hsf is the optical dissector height (7 μm/tissue section thickness. I multiplied this number by two to estimate the total number of neurons in HVC in both hemispheres.

5.2.7 Data analysis

All statistical analyses were performed in SPSS (v. 20, SPSS Inc., Chicago, IL, U.S.A.). I determined the effects of the treatments on singing behavior by conducting one-way ANOVAs with the song attribute of interest (song-type repertoire size, syllable repertoire size, learning accuracy scores, trill deviation, or stereotypy) as the dependent variable and treatment (control, food restriction, CORT) as the independent variable.
Significant main effects of treatment were further analyzed using Fisher’s LSD post-hoc tests. Nest identity (the natal nest of origin) was included in the ANOVAs as a random factor, and paternal song-type repertoire size, paternal syllable repertoire size, and paternal trill deviation were included as covariates. Random factors or covariates that did not significantly affect a given song attribute were removed from the model.

To determine the effects of the treatments on the song control system, I conducted one-way ANOVAs with the brain measurement of interest (the volume of HVC, RA, or area X, number of NeuN+ cells in HVC, or midtelencephalon volume) as the dependent variable and treatment as an independent variable. Significant main effects of treatment were further analyzed using Fisher’s LSD post-hoc tests. Nest identity and brain hemisphere were included as random factors in the ANOVAs and body mass was included as a covariate. In addition, for the analyses on the volumes of HVC, RA, and area X, as well as the number of NeuN+ cells in HVC, I included midtelencephalon volume as a covariate. Nonsignificant random factors and covariates were removed from the analyses. All tests were two tailed and were considered significant at \( p \leq 0.05 \). Data are presented as means ± SEM, adjusted for significant covariates where applicable.

5.3 Results

5.3.1 Song production

I found a main effect of treatment on song-type repertoire size (Fig 5-4A; \( F_{2,19}=4.12, p=0.03 \)). Control subjects had more song types in their repertoires than did
Figure 5-4 The effect of early-life food restriction or corticosterone (CORT) treatment on (A) song-type repertoire size, (B) syllable repertoire size, (C) song-learning accuracy, (D) average trill deviation, and (E) average song-type stereotypy in male song sparrows. Treatments lasted from 7 to 60 days of age. The graph of trill deviation shows the inverse of the minimum orthogonal distance from each of the individual trill types to an upper-bound regression line. Thus a higher score indicates a higher performing trill. *p<0.05, **p<0.01. Data are means ± SEM. SPCC = spectrogram cross correlation.
food-restricted (p=0.04) or CORT-treated subjects (p=0.02), which did not differ from each other (p=0.60). Similarly, I observed a main effect of treatment on syllable repertoire size (Fig 5-4B; F_{2,19}=4.63, p=0.02). Control subjects had more unique syllables in their repertoires than did food-restricted (p=0.01) or CORT-treated subjects (p=0.03). The number of syllables did not differ between food-restricted and CORT-treated birds (p=0.73). For song-learning accuracy scores, (Fig 5-4C), the main effect of treatment was also significant (F_{2,19}=4.60, p=0.02). Control subjects had higher learning accuracy scores than food-restricted subjects (p=0.01). Learning accuracy scores did not differ between control and CORT-treated birds (p=0.29) or between food-restricted and CORT-treated birds (p=0.11). For average trill deviation scores (Fig 4D), the main effect of treatment was not significant (F_{2,16}=2.20, p=0.14). Interestingly, trill deviation score of the experimental birds was negatively related to paternal song-type repertoire size (F_{1,16}=7.08, p=0.02), although not to paternal trill deviation (F_{1,16}=2.22, p=0.16). This indicates that the offspring of males with more song types in their repertoires produced trills with a lower deviation from the upper performance limit and thus higher performing trills. Results for minimum trill deviation scores were very similar: the main effect of treatment (F_{2,16}=1.78, p=0.20) was not significant and paternal song-type repertoire size was again a significant covariate (F_{1,16}=12.24, p=0.003). Lastly, I observed no significant main effect of treatment on either average stereotypy (Fig 5-4E; F_{2,19}=0.01, p=0.99) or, maximum stereotypy (F_{2,19}=0.09, p=0.91).
5.3.2 Song-control system

For the volume of HVC (Fig 5-5A), the main effect of treatment was not significant ($F_{2,19}=1.27, p=0.30$). Body mass was a significant covariate ($F_{1,19}=5.76, p=0.03$); larger birds had a larger HVC. Similarly, for the number of NeuN+ cells in HVC (Fig 5-5B), the main effect of treatment was not significant ($F_{2,20}=0.77, p=0.48$). For the volume of RA (Fig 5-5C), the main effect of treatment was significant ($F_{2,18}=5.54, p=0.01$). The volume of RA was smaller in food-restricted birds than it was in control ($p=0.01$) or CORT-treated ($p=0.01$) birds. The volume of RA did not differ between control and CORT-treated subjects ($p=0.63$). Both body mass ($F_{1,18}=25.36, p<0.001$) and midtelencephalon volume ($F_{1,18}=11.08, p=0.004$) were significant covariates: individuals with a larger body mass and midtelencephalon volume had larger RA volumes. For the volume of area X (Fig 5-5D), the main effect of treatment was not significant ($F_{2,20}=0.32, p=0.73$). Lastly, the main effect of treatment on midtelencephalon volume was not significant ($F_{2,20}=1.61, p=0.22$).
Figure 5-5 The effect of early-life food restriction or corticosterone (CORT) treatment on (A) the volume of HVC, (B) the number of neurons in HVC, (C) the volume of the robust nucleus of the arcopallium (RA), and (D) the volume of area X. Treatments lasted from 7 days of age to 60 days of age. *p<0.01. Data are means ± SEM.

5.4 Discussion

As predicted, exposure to food restriction or CORT treatment during early life had long-term effects on the song of male song sparrows. First, both measures of song complexity were affected. Males exposed to food-restriction or CORT-treatment early in life sang fewer song types and had fewer syllables in their repertoires compared to
control subjects. Food-restricted males also copied the tutor syllables with less accuracy than did control males. However, I found no significant effect of either stressor on either average or maximal measures of vocal performance (song-type stereotypy or trill deviation). Of the three regions of the song-control system that I analyzed (HVC, RA, area X) only RA was affected by developmental stress. The volume of RA was smaller in food-restricted birds than it was in CORT-treated or control birds. Collectively, these findings suggest that early-life stress affects song complexity and song-learning accuracy, two aspects of male song that female song sparrows attend to during mate choice trials (Searcy 1984, Nowicki et al. 2002). Thus, both of these aspects of song may be honest indicators of a male song sparrow’s early ontogeny.

5.4.1 The effects of developmental stress on song

Male song sparrows exposed to food restriction or CORT treatment during development had fewer song types and syllables in their repertoires during adulthood, indicating that early-life stress decreases song complexity in this species. Similarly, both food restriction and CORT treatment were found to decrease syllable repertoire size in a study of zebra finches (Spencer et al. 2003). However, another study of zebra finches showed no effect of food restriction on syllable repertoire size (Brumm et al. 2009). To date, most studies examining the effect of developmental stress on song complexity have been conducted in species with very small song repertoires, such as zebra finches, which sing only one song. Only one previous study has examined the effect of early-life stress on song complexity in a species with a large repertoire (European starlings; Spencer et al. 2004). Consistent with my findings, that study found that European starlings exposed to
an unpredictable food supply during the fledgling period sang fewer song types as adults (Spencer et al. 2004). Thus, developmental food restriction and CORT treatment appear to decrease song complexity by limiting both the number of song types and unique syllables a male sings. In my study, food restriction, although not CORT treatment, also decreased the accuracy with which song sparrows copied the tutor syllables. Similarly, food restriction during the nestling period decreased song-learning accuracy in swamp sparrows (Nowicki et al. 2002b) and zebra finches (Brumm et al. 2009).

In song sparrows, there is some evidence to suggest that song complexity is a sexually selected trait that may influence female mating preferences and/or male-male competition. For example, captive female song sparrows perform more copulation displays in response to high complexity song bouts than they do in response to low complexity song bouts (Searcy 1984). This preference appears to affect mate choice in the field in some populations of song sparrows (Reid et al. 2004), but not all (Searcy 1984). Similarly, female song sparrows also perform more copulation displays in response to songs that contain fewer invented notes (i.e. songs that have been copied more accurately from a tutor; Nowicki et al. 2002b). This suggests that males who are exposed to developmental stress, and that consequently sing less complex songs with poorly copied syllables, may be less attractive to potential mates.

In contrast to song complexity and song-learning accuracy, neither song-type stereotypy nor trill deviation was significantly affected by early-life food restriction or CORT treatment. Song sparrows are closed-ended song learners and as such, cannot compensate for a stressful rearing environment through improving their song complexity or learning accuracy in adulthood: repertoire content remains fixed throughout adulthood.
In contrast, measures of vocal performance, such as stereotypy and trill deviation, may be more dynamic traits that remain plastic throughout adulthood. Indeed, syllable stereotypy changes seasonally in song sparrows and peaks in the spring when HVC volume is greatest (Smith et al. 1997a). It is less clear whether trill deviation is a fixed or dynamic trait (Møller and Pomiankowski 1993). However, this trait does exhibit substantial within-individual variation in Lincoln’s sparrows (*Melospiza lincolnii*; Sockman 2009) and varies with male age in swamp sparrows (Ballentine 2009) consistent with some degree of plasticity. In my study, it is possible that early developmental stress impaired song learning, but that during the months following my experimental treatment the birds were able to develop normal vocal performance. Since features of vocal performance are believed to develop later during the sensorimotor phase of song learning (Podos et al., 2009), which occurs throughout the first year of life in song sparrows, it is possible that exposure to stressors that occur later in development or that encompass a larger portion of the sensorimotor period would affect vocal performance. Thus song complexity and learning accuracy may be more indicative of a male’s early developmental conditions whereas stereotypy and trill deviation may be more indicative of current conditions or conditions experienced later in development. If so, different aspects of song may convey multiple messages with different song features being indicative of the quality of the males’ environment during different stages of their life history (Møller and Pomiankowski 1993).

Trill deviation was the only aspect of song that was related to paternal song production: sons of males with larger song-type repertoires produced trills with lower deviation from the upper performance limit and thus sang higher-performing trills.
Because nestlings were reared in captivity from a very young age, they had extremely limited exposure to their fathers’ songs. This relationship also cannot be attributed to differences in male provisioning ability. Since I found no significant effect of developmental stress on trill deviation, it is possible that this measure of vocal performance may be more strongly influenced by hereditary factors than by environmental factors. In Chapter 2, I found that the total adult body mass and lean mass of the experimental subjects were positively correlated with the mass of their fathers (Schmidt et al. 2012). This suggests that at least some morphological traits are heritable in song sparrows, and it is possible that some sensorimotor abilities are heritable as a result. This raises the intriguing possibility that males that sing more complex song, and that presumably may be of higher phenotypic quality, may pass on genes to their offspring that allow them to produce certain features of song more competently even in the face of a stressful upbringing. However, paternal trill deviation was not related to offspring trill deviation, as might be expected if trill deviation were a heritable trait. My results indicate that some aspects of fathers’ songs are related to the songs of their offspring. However, a clear examination of the heritable aspects of song in these birds is complicated by my experimental manipulation. Lastly, it is also possible that the relationship between paternal song complexity and offspring trill deviation is due to maternal effects. That is, females mated to males with larger song-type repertoires may have invested more in egg production, for example, by producing larger eggs (Leitner et al. 2006) or altering hormone deposition in yolk (Gil et al. 2004), which may influence offspring quality and fitness (Williams, 1994).
5.4.2 The effects of developmental stress on the brain

The only brain region that was significantly affected by developmental food restriction or CORT treatment in my study was RA. The volume of RA was smaller in food-restricted birds than in CORT-treated and control birds. Similarly, in a congeneric species, the swamp sparrow, exposure to food restriction during the nestling period decreased the absolute size of both HVC and RA, but only the relative volume of RA (corrected for telencephalon volume) was significantly reduced (Nowicki et al. 2002a). In a previous study in song sparrows, there was no effect of developmental food restriction on RA volume (MacDonald et al. 2006). However, in that study the stressor was terminated on day 23–26 (at which time birds were euthanized), whereas in my study the stressors continued until day 60. Therefore, in song sparrows, RA may be affected by food restriction later in the fledgling period rather than earlier in the nestling period. In zebra finches, RA begins receiving projections from HVC on day 25 (Mooney and Rao 1994) and undergoes substantial growth between day 25 and day 53, when it nearly doubles in volume (Bottjer et al. 1985; Bottjer et al. 1986). It is possible that RA is particularly susceptible to the effects of stress during this period of rapid maturation. Interestingly, early-life food restriction decreased both the volume of RA and song-learning accuracy. However, there was no effect of developmental stress on the other song nuclei. This finding raises the intriguing possibility that the effect of early-life food restriction on song-learning accuracy may be mediated by alterations in the development of RA. However, as of now this is speculative and future studies are required to determine the role of RA in song-learning accuracy in song sparrows.
I did not detect a significant effect of either stressor on the volume of HVC or area X or on the number of NeuN+ cells in HVC. In contrast, exposing nestling zebra finches to food restriction or CORT treatment reduced the volume of HVC (Buchanan et al. 2004). Also, in a previous study in song sparrows, exposure to food restriction early in development reduced the volume of HVC (MacDonald et al. 2006). However, in that study birds were euthanized immediately after the end of the food restriction period (day 23-26). Therefore, it is possible that the effects of developmental stress on HVC in song sparrows subside once the stressor is terminated. This could be because HVC exhibits a large amount of neural plasticity in adult song sparrows, including large seasonal changes (Smith et al. 1997a; Smith et al. 1997b). Therefore, in song sparrows the size of HVC in adulthood may be more indicative of current conditions, as opposed to developmental conditions. Interestingly, in the present study, HVC volume was significantly correlated with the song features that were not affected by developmental stress including trill deviation (Pearson correlation: $r_{19}=-0.48$, $p=0.026$) and song-type stereotypy ($r_{20}=-0.45$, $p=0.037$), but not to traits that were affected by developmental stress such as song-type number ($r_{20}=0.27$, $p=0.221$), syllable number ($r_{20}=-0.16$, $p=0.489$), or learning accuracy ($r_{20}=-.11$, $p=0.62$). This further supports the hypothesis that measures of vocal performance may be indicative of current conditions that affect seasonal changes in HVC, as opposed to developmental conditions. In adult song sparrows, chronic treatment with CORT decreases the volume of HVC as well as the number of proliferating cells in HVC (Newman et al. 2010). An interesting future study would be to determine whether measures of vocal performance are affected by administration of CORT or exposure to
stressors in adulthood, and whether vocal performance is significantly related to indices of physiological stress (e.g. HPA regulation, heterophil levels) in free-living individuals.

### 5.4.3 Differential effects of food restriction and CORT

Food restriction affects brain development and subsequently alters behaviour and cognition in humans (Laus et al. 2011), rats (Gressens et al. 1997), and songbirds (Buchanan et al. 2004). There are many potential mechanisms that could be responsible for the effects of food restriction on development. One hypothesis is that food restriction may increase levels of glucocorticoids, which in turn may alter development of the brain (Welberg and Seckl 2001). Food restriction can increase baseline and stress-induced levels of plasma glucocorticoids in songbirds (Kitaysky et al. 2001; Kempster et al. 2007) and mammals (Lesage et al. 2001). In addition, consistent with previous studies of zebra finches (Spencer et al. 2003; Buchanan et al. 2004), I found that both food-restriction and CORT treatment decreased song complexity in song sparrows, suggesting that CORT may mediate the effects of food restriction on song complexity. In contrast, only food-restriction decreased song-learning accuracy and the volume of RA, suggesting that an increase in CORT does not mediate the effects of food restriction on these traits.

In addition to an increase in glucocorticoids, there are several other potential mechanisms that could explain the detrimental effects of food restriction on the brain. For example, food-restricted individuals may have fewer nutrients and substrates to support brain development. In particular, low protein levels during the prenatal and postnatal period have many detrimental effects on the developing rodent brain, including a decrease in the density of blood vessels (Bennis-Taleb et al. 1999), reduced brain
metabolism (Gallagher et al. 2005), and alterations in the composition and quantity of myelin (Simons and Johnston 1976). In addition, food-restricted individuals may face trade-offs in the development of different physiological systems if nutrients are limited (Gluckman et al. 2005). For example, early-life food restriction may initially retard growth but be followed by a period of accelerated growth (called catch-up growth) once food restriction ends. Catch-up growth may allow individuals to attain normal adult body size, but may take resources away from the development of other systems leading to long-term physiological impairments (Hales and Ozanne 2003; Metcalfe and Monaghan 2001). In Chapter 2, I found that CORT-treated individuals were structurally smaller at day 45 compared with control individuals, but that food-restricted birds were similar in size to control birds (Schmidt et al. 2012). Therefore, food-restricted birds may have traded off development of the brain in order to allocate resources to somatic growth. Lastly, it is also possible that food restriction decreased song-learning accuracy because males in this group may have spent more time sleeping to conserve energy, or more time searching for food, and as a result may have spent less time actively listening to the tutor song or producing sub-song during the song-learning period.

5.4.4 Conclusion

My results provide support for the Developmental Stress Hypothesis and show that early-life food restriction and CORT treatment affect song complexity and song-learning accuracy in a species with a complex repertoire. In addition, early-life food restriction affected the volume of RA suggesting that developmental stress can have long-lasting effects on the song-control system in song sparrows. Thus the effects of
developmental food restriction on song-learning accuracy in song sparrows may be mediated by alterations in the development of RA. Not all features of song were affected by early-life food restriction or CORT treatment in my study, suggesting that some song features, such as measures of vocal performance, may be more indicative of a male’s current or recent conditions as opposed to the conditions he faced during early development. In addition, trill deviation was significantly related to paternal song complexity suggesting that variation in this trait may be largely explained by heritable factors. Since stress has widespread effects on the development of other physiological systems, exposure to early-life stress may be one mechanism by which song comes to be a reliable indicator of a variety of adult traits, including traits that are not functionally related to song (Spencer and MacDougall-Shackleton S.A. 2011).


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Chapter 6

6 Early-Life Stress affects the Behavioural and Neural Response of Female Song Sparrows to Conspecific Song

6.1 Introduction

Stress experienced early in life can alter brain development leading to long-term effects on cognition and behaviour in many vertebrate species (Welberg and Seckl, 2001). In songbirds, developmental stress can have persistent effects on multiple aspects of cognition and behaviour including spatial learning (Farrell et al., 2011), social dominance, and neophobia (Spencer and Verhulst, 2007). In addition, a variety of early-life stressors can impair development of the brain regions that are important for song learning and production and affect adult song production (Nowicki et al., 1998). For example, food restriction affects growth of the song nucleus HVC in zebra finches (Taeniopygia guttata; Buchanan et al., 2004) and song sparrows (Melospiza melodia; MacDonald et al., 2006; but see Chapter 5). Male zebra finches exposed to early-life food restriction or treatment with the glucocorticoid hormone corticosterone (CORT) also sing songs that are shorter and less complex (Spencer et al., 2003). Swamp sparrows (Melospiza georgiana) exposed to food restriction early in life produce poorer copies of their tutor’s song compared to males exposed to control conditions (Nowicki et al., 2002a). Since song is a sexually selected trait that is important for attracting mates and/or inter-male aggression in many species (Collins, 2004), the effects of early-life stress on
song-control nuclei and subsequent song production could have important fitness consequences for songbirds.

In most species of songbirds that breed in the temperate-zone, males sing much more than females, if females sing at all. However, even if females do not produce song, they may attend to a variety of features of male songs when selecting mates (Nowicki and Searcy, 2005). For example, females may prefer songs that are more complex (Reid et al., 2004), that are more similar to those of their natal population (MacDougall-Shackleton et al., 2001), or that were more accurately learnt from a tutor (Nowicki et al., 2002b).

Although there is now substantial evidence that developmental stressors can have long-term effects on song learning and production in males (reviewed in MacDougall-Shackleton and Spencer, 2012), few studies have examined the effects of developmental stress on female song preferences. However, in some species (including song sparrows; Hernandez et al., 2009), exposure to songs during development affects female song preference in adulthood, indicating that females learn or memorize songs they later prefer. Therefore, since early-life stress has been shown to affect song learning in males, it is possible that early-life stress may affect learned female song preferences. So far, studies testing this hypothesis have found mixed results. In one study, female zebra finches raised in experimentally enlarged broods (and presumably exposed to developmental stress) had weaker preferences than did control females when choosing between two unfamiliar songs (Riebel et al., 2009). Interestingly, another study of zebra finches found that females raised in large broods preferred the songs of males raised in similar conditions, while females raised in small broods preferred songs of males from small broods, potentially resulting in assortative mating (Holveck and Riebel, 2010).
These results suggest that early-life stress may affect both the strength and direction of female song preferences. However, there appears to be no effect of early-life food restriction on the preference for high complexity songs in zebra finches (Woodgate et al., 2011). Therefore, the effects of developmental stress on female preference may depend on the type of stressor, as well as the particular aspect of song being tested.

Despite a growing body of evidence implicating developmental stress in adult song preferences, the neural bases underlying these effects are unclear. Two descending pathways of song control nuclei have been identified as important for song learning and production (Margoliash, 1997). The development of two nuclei in particular, HVC and the robust nucleus of the arcopallium (RA), may be impaired in individuals exposed to early-life stress (Nowicki et al., 2002a; Buchanan et al., 2004). These two nuclei are important for the learning or production of song in males. However, studies investigating the role of the song-control nuclei in the formation or maintenance of female song preferences have found conflicting results. For example, female canaries (Serinus canaria domestica) that are better able to discriminate between songs of different quality have a larger HVC (Leitner and Catchpole, 2002), and lesions of HVC disrupt normal song preferences in this species (Brenowitz, 1991). However, in female zebra finches, lesions of HVC had no effect on the preference for conspecific song over heterospecific song (MacDougall-Shackleton et al. 1998a).

In addition to the song-control nuclei, a series of auditory regions in the forebrain connect the general auditory area, field L (homologous to primary auditory cortex of mammals), to the song-control system (Vates et al., 1996). In contrast to studies investigating the role of the song-control control nuclei, there is strong evidence that
these auditory forebrain regions are important for song discrimination and the formation and maintenance of song preferences. For example, lesions to one of these regions, the caudomedial mesopallium (CMM) impair the preference of female zebra finches for conspecific over heterospecific song (MacDougall-Shackleton et al., 1998a). In addition, these regions, including CMM and the caudomedial nidopallium (NCM), exhibit enhanced induction of the immediate early gene ZENK (an acronym for Zif-268, Egr-1, NGFIA, and Krox-24) when birds hear song but not when they produce song (Jarvis and Nottebohm, 1997). The ZENK gene encodes a protein (referred to here as Zenk), which is a transcription factor mediating the effects of growth factors and other signals on membrane depolarization. Zenk has been widely used in songbirds to investigate the neural response to song presentation (Tischmeyer and Grimm, 1999; Hernandez et al., 2008). For example, Zenk induction is increased in male zebra finches and canaries exposed to conspecific song compared to males exposed to silence, a pure tone, or heterospecific song (Mello and Clayton, 1994). In addition, female white-crowned sparrows (Zonotrichia leucophrys) show higher Zenk induction in CMM and NCM in response to their local song dialect compared to a foreign dialect, and also show a stronger behavioural response to the local dialect (Maney et al., 2003). Moreover, individual variation in Zenk induction was correlated to levels of courtship behaviour in this study (Maney et al., 2003). To date, no studies have determined the effects of early-life stress on Zenk induction in the auditory forebrain regions of the songbird brain. Thus, the hypothesis that changes in the behavioural response to song are mediated by changes in the neural responsiveness of the auditory forebrain regions to song presentation remains an open question.
In the current study, I examined the long-term effects of developmental stressors (either food restriction or exogenous CORT treatment) on 1) the behavioural response to high-complexity versus low-complexity songs, and to conspecific versus heterospecific songs; 2) the volume of the song-control nuclei HVC and RA, and 3) the number of cells that were immunoreactive for the immediate early gene Zenk in CMM and NCM following exposure to conspecific or heterospecific song in female song sparrows. In song sparrows, only males sing and a bird typically sings 5-12 unique song types (Pfaff et al., 2007). In addition, song sparrows are closed-ended song learners, meaning that males do not alter their song repertoires in adulthood (Nordby et al., 2002). Female song sparrows show a preference for high complexity song bouts in laboratory studies (Searcy, 1984). In addition, the preference of female song sparrows for locally typical song is influenced by sensory experience early in life (Hernandez et al., 2009) suggesting that the formation of song preferences may be susceptible to variation in the early-rearing environment in this species. To my knowledge, this is the first study to examine the long-term effects of chronic early-life stress on induction of an immediate early gene in the brain of any species. I predicted that females exposed to early-life food restriction or CORT treatment would: 1) be less selective in their behavioural response to high-complexity versus low-complexity song and less selective to conspecific versus heterospecific song than control females, 2) have smaller song-control nuclei than control females, and 3) have similar levels of Zenk in auditory forebrain regions after exposure to conspecific or heterospecific song, whereas control females would have higher levels of Zenk to conspecific song.
6.2 Methods

6.2.1 Subjects

The female subjects used in this study were the same as the female subjects used in Chapter 2 (Schmidt et al., 2012a). However, due to mortality sample sizes differed slightly: control n=7, food restriction n=7, CORT n=8 (Table 6-1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample size</th>
<th>Euthanized after exposure to conspecific song</th>
<th>Euthanized after exposure to heterospecific song</th>
<th>Age at start of tutoring (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>20.71 ± 2.77</td>
</tr>
<tr>
<td>Food Restriction</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>18 ± 2.40</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>8</td>
<td>3</td>
<td>5</td>
<td>16.13 ± 2.45</td>
</tr>
</tbody>
</table>

6.2.2 Early-life song exposure

Females were exposed to song because exposure to song during development affects the preference of female song sparrows for song later in life (Hernandez et al., 2009). All birds were housed in one room and therefore had identical exposure to song early in life. Birds were exposed to recorded male song sparrow song beginning at d5-d34 (mean=18.18 days, SEM=1.45) and lasting until d11-d140 (mean=124.18,
SEM=1.45). Although the age of individuals at the start and end of the song-tutoring period varied, all birds were exposed to recorded song for exactly 106 days. In addition, the average age of birds at the start of the song-tutoring period did not differ between treatment groups (Table 6-1). In male song sparrows, song acquisition occurs primarily from 10-50 days of age and can extend up to 200 days, however 90% of songs are acquired by 90 days post-hatch (Marler and Peters, 1987). In addition, because there is evidence that song learning may be enhanced by exposure to live tutors (Beecher and Burt, 2004), 4 adult males were captured near the area where nests were located and were housed in the same room as the experimental subjects. These 4 males were placed on opposite corners of the room and their location was changed every two weeks so that all subjects had similar exposure to each male. Song was not quantified from the live tutors, however one male was observed singing frequently and the other three were never heard singing.

Song exposure stimuli were prepared using Syrinx software (version 2.6h; J Burt; www.syrinxpc.com). Male song sparrows from my population typically sing between 5-12 unique song types (Pfaff et al., 2007; Schmidt et al., 2012b). Tutor playback sequences consisted of 32 unique song types from 4 individual males. These males were recorded in the summer of 2010 from the same population as the experimental birds. The recorded males had territories that were at least 1 km away from the subjects’ home nests. Song recordings of high quality (based on signal-to-noise ratio and the absence of background sound) were selected for use as tutor stimuli. Each song type was filtered to remove background noise and then peak amplitude was equalized. This number of tutor singers and exemplars was chosen as representative of the number of singing individuals
that a young song sparrow is likely to be exposed to in the wild. Two different song playback sequences were created that contained the 32 unique song types in random order. Song sparrows sing with eventual variety, meaning that they sing one song type several times before switching to a new song type. Accordingly, each song type was repeated 8 times on the playback before switching to the next song type. Songs were played at a rate of 6 songs per minute, which mimics natural song rate. Thus, each playback contained the 32 song types, each one repeated 8 times (total of 256 songs over 43 min). Each playback was played once in the morning, afternoon, and evening with an hour of silence between the two playbacks. Thus birds were exposed to 258 min (43 min x 6) of song per day.

6.2.3 Behavioural testing procedure

Birds were kept on a long day photoperiod (16L:8D) from the time they were brought into captivity until 16 August 2010, at which time they were switched to short days (10L:14D) for 18-21 weeks. This was done to simulate winter conditions in order to bring birds into reproductive condition at the start of behavioural testing. Birds were then put back on long days (16L:8D) 5 weeks before behavioural testing began. I did not confirm reproductive condition at the start of the behavioural testing because all birds received implants of 17β-estradiol (see below) and therefore should have been receptive regardless of reproductive condition. However, I visually inspected the ovaries at the end of the behavioural testing after birds were euthanized. In all subjects, the ovaries had begun to regress (likely due to negative feedback induced by the estradiol implants) but
were not yet completely regressed and either had a slightly granular appearance or contained small follicles. No individuals had large or yolky follicles.

I conducted two separate behavioural experiments (Fig 6-1). Study 1 (Fig 6-1B) compared the response of females to high complexity versus low complexity conspecific song and study 2 (Fig 6-1C) compared the response of females to conspecific versus heterospecific song. Details of song stimuli for behavioural tests are provided below. Testing began when birds were ~9 months of age (mean=265.32 days, SEM=0.99), which was ~7 months after the end of the experimental treatments. For both experiments, females were housed individually in cages that contained a food and water dish and two perches placed in the same location in all cages.

**Study 1: High versus low complexity song**

Prior to study 1 (Fig 6-1B), birds received a subcutaneous implant of 17β-estradiol in silastic tubing (inner diameter=1.47 mm, outer diameter=1.96 mm, length=12 mm) on their upper back. Implants were sealed with medical adhesive (Dow Corning, Midland, MI, USA). Estradiol implants are necessary to prime female song sparrows to perform copulation solicitation displays in captivity (Searcy and Marler, 1981; Searcy and Capp, 1997). Four days after birds were implanted, they were moved to sound attenuation chambers (Eckel Noise Control Technologies, Morrisburg, ON, Canada, model AB-2000 S). Testing began 8 days after birds were implanted with estradiol. I tested females on two separate days with one day in between. On each day of testing, a female was exposed to a low complexity song bout and a high complexity song bout with 3 h between trials (see below for details of stimulus preparation). On the first day of
testing, half of the females in each treatment group heard low complexity song first and half heard high complexity song first. The order of presentation was then reversed on the second day of testing. Songs were played at constant amplitude through a speaker (Optimus Pro-X44AV) that was placed adjacent to the bird’s cage out of their field of view. The behavioural response of females was recorded using a video camera mounted on a tripod. Testing began 30 min after the camera was placed in the chamber and was completed between 8 AM – 2 PM.

**A. Experimental Timeline**

```
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<td>Treatment</td>
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**B. Study 1: high Complexity versus low complexity song**

```
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<td>Testing day 1</td>
<td>Rest</td>
<td>Testing day 2</td>
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<td>Trial 1</td>
<td>Trial 2</td>
<td>Trial 1</td>
<td>Trial 2</td>
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</tr>
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</table>
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**C. Study 2: conspecific versus heterospecific song**

```
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<th>day 4</th>
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</thead>
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<td>Back to chambers</td>
<td>Testing day 1</td>
<td>Rest</td>
<td>Testing day 2</td>
</tr>
<tr>
<td>Brain collection</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
```

**Figure 6-1** Timeline of (A) the whole experiment, (B) Study 1, and (C) Study 2

In pilot trials, females performed few, if any, copulation solicitation displays. In an attempt to increase the response of females to the songs, a male song sparrow in
nonbreeding condition was placed in a cage adjacent to the females during all playback trials in study 1. An opaque divider was placed between the two cages with a small space on one end so that females could see the male if they looked around the divider. Two males were used and the same male was used for both testing trials within a day. This ensured that any difference in behavioural response to high complexity versus low complexity song was due to the different song stimuli and not to the male. The males occasionally vocalized but did not produce full songs during behavioural trials. At the end of testing, birds were removed from the sound attenuation chambers and housed in individual cages in a holding room until the start of study 2.

*Study 2: conspecific versus heterospecific song*

In study 2 (Fig 6-1C), I compared the behavioural and neural response of females to conspecific versus heterospecific (Gambel’s white-crowned sparrow, *Zonotrichia leucophrys gambelii*) song. Three weeks after testing for study 1, birds were moved back to the sound attenuation chambers and allowed to habituate for two days before testing. Estradiol implants were still in place for study 2. Females were exposed to one conspecific and one heterospecific song bout on separate days of testing with one day in between. Order of presentation was counterbalanced such that half of the females from each group heard conspecific song first and the other half heard heterospecific song first. A male was not placed next to females during testing in study 2. Songs were played at constant amplitude from a speaker and behaviour was recorded using a video camera mounted on a tripod. Testing began 30 min after the camera was placed in the chamber and was completed between 9 AM – 1 PM.
On the second day of testing, females were euthanized after exposure to either conspecific or heterospecific song (Table 6-1). Immediately after the song stimulus ended, the lights in the chamber were turned off and birds were left in the dark and quiet for 1 h. Birds were then euthanized with an overdose of isoflurane and brains were rapidly extracted. The left and right hemispheres were bisected using a scalpel blade. One hemisphere was immediately frozen on powdered dry ice to be used in another study. The remaining hemisphere was post-fixed in 5% acrolein for 1.5 h. Within a treatment group, I used the left hemisphere for half of the subjects and the right hemisphere for the remaining half of the subjects. Brains were then washed 3 times in 0.1 M phosphate buffered saline (PBS) for 30 min before being transferred to 30% sucrose for cryoprotection. The following day brains were rapidly frozen on powdered dry ice and stored at -80 °C until processing.

6.2.4 Song stimuli for behavioural testing

Song playbacks were created using Syrinx software. All songs were filtered to remove background noise and peak amplitude was equalized.

*Study 1: high versus low complexity song*

I selected 12 high quality male song sparrow songs from 3 individuals that females had previously been exposed to during the early-life song exposure period. For high complexity song bouts, females heard all 12 song types played in random order, each one repeated 8 times before switching to the next song type. Songs played at a rate of 6 songs per min. For low complexity song bouts, females heard 4 of the 12 song types (selected randomly) each one repeated 8 times. This set of 4 song types was played three
times so that the high complexity and low complexity song bouts consisted of the same number of songs (96) and played for the same amount of time (16 min). Females were exposed to 4 different song types for each of the two low complexity trials (8 of the 12 in total). In the wild, males usually sing 5-12 song types, but occasionally may sing as few as 4 (Reid et al., 2004). Therefore, 12 song types represents the upper range of normal for a free-living male song sparrow and 4 the lower range of normal.

**Study 2: conspecific versus heterospecific song**

Conspecific song playback consisted of 6 unique song types from 6 male song sparrows (Fig 6-2A). These songs were recorded from male song sparrows in the summer of 2010 from the same population as the experimental subjects but birds had not been exposed to these songs during the early-life song exposure period and these songs were thus unfamiliar. Heterospecific song playback consisted of 6 unique song types from 6 male Gambel’s white-crowned sparrows (Fig 6-2B). White-crowned sparrows were chosen because their songs are similar in length and pitch to those of song sparrows. In addition, although song sparrow songs contain a larger variety of syllable types than songs from white-crowned sparrows, songs from the two species are similar in frequency bandwidth and both contain complex trills and buzzes (Fig 6-2). These individuals were recorded in Davis, California, USA in March 2010. For both conspecific and heterospecific playbacks, females were exposed to all 6 song types (played in random order). Each song type was repeated 32 times at a rate of 6 songs per min. Therefore, conspecific and heterospecific playbacks did not vary in the number of unique song types (6), the number of total songs played (192), or in total length (32 min).
**Figure 6-2** Spectrograms of (A) three song sparrow songs and (B) three Gambel’s white-crowned sparrow songs used to determine the behavioural and neural response to conspecific (song sparrow) versus heterospecific (white-crowned sparrow) song.

### 6.2.5 Immunocytochemistry

Brains were sectioned in the sagittal plane at 40 μm thickness using a cryostat. I collected two sets of sections into 0.1 M phosphate buffered saline (PBS), the first was used for Zenk immunoreactivity and the second for NeuN immunoreactivity. NeuN is a protein that is expressed in the majority of neuronal cell types (Mullen et al., 1992) and was used to calculate the volumes of the song nuclei HVC and RA (see below). In each
run (11 runs total), I completed Zenk and NeuN immunocytochemistry (ICC) for two individuals from different treatment groups. First, free-floating sections were washed 2x in 0.1 M PBS, followed by incubation in 0.5% hydrogen peroxide. Sections were further washed 3x in PBS and then incubated for 1 h in 10% normal goat serum (Vector, Burlingame, CA, U.S.A.) diluted in 0.3% Triton in PBS (PBST). For both Zenk and NeuN ICC, sections were then incubated for 20 h in primary antibody diluted 1:2000 in 0.3% PBST. For Zenk, I used a polyclonal antibody (Egr-1 Sc-189, Santa Cruz Biotechnology, Dallas, TX, U.S.A.) raised in rabbit (Hernandez and MacDougall-Shackleton, 2004). For NeuN, I used a monoclonal antibody (MAB377, EMD Millipore Corporation, Billerica, MA, U.S.A.) raised in mouse (Phillmore et al., 2006; Newman et al., 2010). Sections were then washed 3 times in 0.1% PBST and incubated for 1 h in biotinylated secondary antibody diluted 1:250 in 0.3% PBST (goat anti-rabbit IgG for Zenk and goat-anti mouse IgG for NeuN, both from Vector, Burlingame, CA). After 3 washes in 0.1% PBST, sections were treated with avidin-biotin horseradish-peroxidase complex (Vector Elite ABC kit) for 1 h and then washed 2 times in 0.1% PBST. Sections were immediately visualized by exposing them to diaminobenzidine solution (Sigma Fast-DAB) and washed 3 times in PBS. Sections were mounted onto gelatin-coated slides, dehydrated with a series of graded ethanols, and cleared in solvent (Harleco Neo-Clear, EMD Chemicals). Lastly, slides were cover-slipped using Permount (Fisher Scientific).
6.2.6 Song nuclei volumes

Using a Leica Digital CCD camera mounted on a Leica DM5000B light microscope (Leica Microsystems inc. Richmond Hill, ON, Canada), I captured images from every NeuN labeled section that contained the song nuclei HVC and RA. I then used Leica Application Suite software to trace the area of the regions of interest and combined the area measurements using the formula for the volume of a frustrum to calculate the volume of HVC and RA in one hemisphere. Measurements were then multiplied by two to estimate the total volume of each region in both hemispheres. Song nuclei volumes do not differ between the left and right hemispheres (Tramontin et al., 2000). In order to obtain an index of total brain size, slides were scanned at 2,400 dpi into a computer using a flatbed scanner with a transparency adapter. Using imageJ64 software (National Institutes of Health, Bethesda, MD) I then measured the area of the total telencephalon for 11 sections centered on the largest cross section of HVC. Area measurements were combined using the formula for the volume of a frustrum to produce a measure of midtelencephalon volume (as in MacDougall-Shackleton et al., 1998b).

6.2.7 Zenk quantification

I used a quantification protocol similar to several previous studies (Gentner et al., 2000; Maney et al., 2003; Hernandez and MacDougall-Shackleton, 2004; McKenzie et al., 2006). I quantified Zenk immunoreactivity in 3 regions within the telencephalon (Fig 6-3) that show enhanced immunoreactivity in response to conspecific song (Mello and Clayton, 1994; Gentner et al., 2000; Maney et al., 2003): CMM, dorsal NCM (dNCM), and ventral NCM (vNCM). These two regions of NCM have previously been shown to
exhibit differential Zenk induction in response to playback (Gentner et al., 2000; McKenzie et al., 2006).

Figure 6-3 Sagittal section of the female song sparrow brain showing the regions where Zenk immunoreactivity was quantified. The section shown was from a subject reared under control conditions that was euthanized after exposure to conspecific song. The rectangular boxes show the approximate areas sampled within each region. CMM = caudomedial mesopallium, dNCM = dorsal caudomedial nidopallium, vNCM = ventral caudomedial nidopallium. Field L2 is evident as the area lacking immunoreactivity.

Moving medially to laterally, I began quantification when NCM became attached to the rest of the brain and field L2 was visible as an area without Zenk immunoreactivity.
(Mello and Clayton, 1994). I quantified 6 sections per region per bird (Maney et al., 2003). Since alternate 40 µm sections were analyzed, I sampled a total area that was 480 µm wide mediolaterally, similar to previous studies (Maney et al., 2003). The specific sampling location within each region was chosen centrally within the structure to ensure I was sampling well within the border of each region. I was blind to both the treatment group and song playback condition of the bird. Images of each sampling location (0.515 mm x 0.386 mm) were captured using a Leica Digital CCD camera mounted on a Leica DM5000B light microscope through a X20 objective lens. I then used ImageJ64 software to count the number of Zenk immunoreactive cells in the entire image. Images were converted to 8-bit grayscale images and then the number of particles with an optical density above a threshold value were counted using the threshold feature in ImageJ. Because of variability in background staining, this threshold was set manually for each image such that clusters of pixels highlighted by the software matched with what a blind observer considered labeled nuclei (Maney et al., 2006). I set exclusion limits for cell size by calculating the size of 50 cells (2-3 cells per individual from randomly chosen sections). Maximum cell size was set as one standard deviation above this value and minimum cell size as one standard deviation below. Exclusion limits for sphericity were set at 0.65.

6.2.8 Data analysis

Statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Initially, I intended to use the number of copulation solicitation displays performed as the sole dependent measure for behavioural studies. However, only 6 of the
22 females performed copulation displays so I also measured activity levels (number of hops) as a measure of the behavioural response to song. Number of hops has been used to investigate the behavioural response of females to song in several previous studies (Baker et al., 1982; Woodgate et al., 2010). I used Jwatcher (version 1.0; www.jwatcher.ucla.edu) to score the number of copulation displays and hops. I scored all behavioural videos and was blind to the treatment group and playback condition of the birds. This was achieved by scoring the videos with the sound turned off.

For study 1, I added the number of displays and hops performed for the two low complexity trials and for the two high complexity trials. For both study 1 and study 2, I calculated a behavioral response score for each female that allowed me to directly compare both the direction and strength of their behavioural response. This was done by calculating the proportion of total hops that were made in response to high complexity song (study 1) or to conspecific song (study 2) following Woodgate et al., (2011). I then determined if the behavioural response scores were significantly different from 0.50 (no selectivity in response) for each treatment group using one sample t-tests. In addition, to directly compare the behavioural response scores between the three treatment groups I conducted a one-way ANOVA with behavioural response score as the dependent variable and treatment as the independent variable. Nest identity (the natal nest that birds came from) was initially included as a random factor, but was not significant and was therefore removed from the analysis. Main effects of treatment were analyzed using Fisher’s LSD post-hoc test. Lastly, to compare overall activity levels between treatment groups in both study 1 and study 2, I compared the total number of hops using a two-way repeated measures ANOVA with treatment as a between-subject factor and study (1 or 2) as a
repeated-measures factor. One female in the control condition either did not hop or
display or made very few hops in multiple trials and was excluded from the analyses. In
addition, for study 2, one female was inadvertently exposed to conspecific song twice so
this subject was excluded from analyses for study 2.

For study 1, I also used a paired t-test to compare the number of copulation
solicitation displays performed in response to high complexity versus low complexity
song bouts, for the 6 subjects that did perform displays. In addition, in order to determine
if hopping behaviour is a reasonable proxy for sexual response in this species I
determined if the number of hops was related to the number of copulation displays for the
6 individuals that displayed using a linear mixed model. This model included the number
of displays and hops performed for both the low complexity and high complexity trials,
thus each individual was represented twice. Therefore, I included subject identity as a
random variable and the number of copulation displays was set as the dependent variable.
The number of hops was added as a continuous covariate and then entered into the
analysis as a main effect. This analysis was only conducted for study 1, because even
fewer individuals performed displays during study 2.

To determine the effects of the treatments on HVC and RA volume I conducted a
linear mixed model using a restricted maximum likelihood (REML) model. I included
subject as a random variable, region (HVC or RA) as a repeated factor, and treatment as a
fixed factor. Initially, hemisphere (left or right) and nest identity were added as random
factors and midtelencephalon volume was added as a covariate, but these factors were not
significant and were therefore removed from the analysis. We also determined the effect
of the treatments on midtelencephalon volume (an index of overall brain size) using an
ANOVA with midtelencephalon volume as the dependent variable and treatment as the independent variable.

I also used linear mixed models to analyze the number of Zenk immunoreactive cells in the brain. The total number of Zenk immunoreactive cells in each region was included as the dependent variable. Brain region (CMM, dNCM, vNCM) was included as a repeated factor and treatment and playback (conspecific or heterospecific) were included as fixed effects. Hemisphere and nest identity were added as random factors, but were not significant and were therefore removed from the analysis. The initial model included the three-way interaction (treatment x playback x region) and all of the two-way interactions, but I removed nonsignificant interaction terms from the analysis in order to create the simplest model possible. The final model included the main effects of brain region, treatment, and playback, as well as the treatment x playback interaction. The significant treatment x playback interaction was further analyzed by conducting linear mixed models for each treatment with region as a repeated factor and playback condition as a fixed factor. All tests were two-tailed and were considered significant at $p \leq 0.05$.

6.3 Results

6.3.1 Behavioural testing

*Study 1: high versus low complexity song*
For study 1, mean behavioural response scores (Fig 6-4A) were significantly above chance (0.50) for control subjects only, although there was a trend for food-restricted birds (control: \( t_5 = 3.82, p = 0.01 \); food restriction: \( t_6 = 2.30, p = 0.06 \); CORT: \( t_7 = 1.53, p = 0.17 \)). There was no significant difference in behavioural response scores between the 3 treatment groups (\( F_{2,18} = 0.28, p = 0.76 \)). However, although there was no significant difference between groups in the strength of behavioural response, only the control group had behavioural response scores that differed significantly from chance. This was likely due to reduced variance in this group (Fig 6-4A).

For the total number of hops performed (Fig 6-5), the main effect of treatment was not significant (\( F_{2,18} = 1.78, p = 0.20 \)) nor was the treatment x study interaction (\( F_{2,18} = 0.07, p = 0.93 \)), indicating that there was no significant difference in overall activity levels between the 3 treatment groups for either study.

In study 1, 6 individuals (1 control, 2 food-restricted, and 3 CORT-treated subjects) performed copulation solicitation displays. These individuals performed more copulation displays to high complexity song bouts than low complexity song bouts (Fig 6-6; \( t_5 = 2.66, p = 0.04 \)), consistent with a stronger preference for high complexity song. For these individuals, the number of hops performed in response to song playback was positively related to the number of copulation displays for a given trial (Fig 6-7; \( F_{1,9.20} = 7.79, p = 0.02 \)), suggesting that hopping behaviour is a reasonable proxy for sexual response in this species.
Figure 6-4 The response of female song sparrows raised in three different treatment conditions to (A) high complexity versus low complexity conspecific song (study 1) and (B) conspecific versus heterospecific (Gambel’s white-crowned sparrow) song (study 2). Behavioural response scores were calculated as the proportion of total hops that were made for high complexity (study 1) or to conspecific (study 2) song, where a value of 0.5 indicates no selectivity in response. *p<0.05, **p<0.01, +p=0.06. Data represent means ± SEM. Asterisks directly above a bar indicate that mean behavioural response was significantly different from 0.5. CORT = corticosterone
Figure 6-5 The total number of hops performed by female song sparrows raised in three different treatment groups for both study 1 (response to high complexity versus low complexity song bouts) and study 2 (response to conspecific versus heterospecific song). Data represent means ± SEM. CORT = corticosterone.

Figure 6-6 The number of copulation solicitation displays performed by female song sparrows in response to high complexity versus low complexity song bouts, n = 6. Data represent means ± SEM. *p<0.05.
Figure 6-7 The relationship between the number of hops and the number of copulation solicitation displays performed by female song sparrows in response to song in study 1 (response to high complexity versus low complexity conspecific song bouts). The graph shows data from both the high complexity and low complexity trials for 6 individuals that performed copulation displays.

Study 2: conspecific versus heterospecific song

For study 2, mean behavioural response scores (Fig 6-4B) were significantly above 0.50 for control subjects only (control: t₅=3.60, p=0.02; food restriction: t₆=0.65, p=0.54; CORT: t₆=0.76, p=0.76). There was a significant difference in behavioural response scores between treatments (F₂,₁₇=5.15, p=0.02). Control subjects were more selective in their behavioural response to conspecific versus heterospecific song (higher proportion of total hops made to conspecific song) than subjects exposed to early-life stress (control versus food restriction: p=0.01; control versus CORT: p=0.04; food restriction versus CORT: p=0.36).
6.3.2 Song-control nuclei

I observed no significant main effect of treatment on the size of the song-control nuclei HVC and RA (Fig 6-8; $F_{2,19.08}=1.10$, $p=0.35$), nor any significant treatment x brain region interaction ($F_{2,19.21}=0.25$, $p=0.79$). The main effect of brain region was significant ($F_{2,19.23}=35.55$, $p<0.001$): HVC was larger than RA. There was no difference in midtelsonchalon volume between treatment groups ($F_{2,19}=0.26$, $p=0.78$).

6.3.3 Zenk immunoreactive cells

Predictors of Zenk immunoreactivity entered into the final model included main effects of treatment (control, food-restricted or CORT), playback (conspecific or heterospecific), and brain region (CMM, dNCM, vNCM), as well as the treatment x playback interaction. The main effect of brain region was not significant ($F_{2,41.06}=0.63$, $p=0.54$), nor was the main effect of treatment ($F_{2,55.97}=0.54$, $p=0.58$). The main effect of playback was significant ($F_{2,55.97}=4.96$, $p=0.03$). However, the treatment x playback interaction was also significant ($F_{2,55.97}=3.27$, $p=0.045$), indicating that the effect of playback depended on the experimental treatment. Moreover, exclusion of this interaction term reduced the overall explanatory power of the model (Akaike’s information criterion [AIC] value excluding interaction $=868.16$; AIC value including interaction $=838.049$). Therefore, to account for this interaction, I conducted linear mixed models for each treatment group that included the main effects of playback and brain region as well as the two-way interaction. For control subjects (Fig 6-9A), neither the playback x brain region interaction ($F_{2,11.54}=0.14$, $p=0.87$), nor the main effect of brain region ($F_{2,11.54}=1.07$, $p=0.38$) were significant. However, the main effect of playback was significant...
Across all brain regions, the number of Zenk immunoreactive cells was higher in individuals exposed to conspecific song compared to individuals exposed to heterospecific song (Fig 6-9A). For subjects exposed to early-life stress (Fig 6-9B and 6-9C), neither the playback x brain region interaction (food restriction: F\(_{2,10.50}=0.22, p=0.81\); CORT: F\(_{2,12.63}=0.52, p=0.61\)), nor the main effects of brain region (food-restriction: F\(_{2,10.50}=0.06, p=0.94\); CORT: F\(_{2,12.63}=0.03, p=0.97\)) or playback (food restriction: F\(_{2,13.39}=0.17, p=0.69\); CORT: F\(_{2,17.10}=1.42, p=0.25\)) were significant. However, when analyzing each treatment group separately, sample sizes were low when comparing individuals exposed to conspecific versus heterospecific song (Table 6-1), which would make it difficult to detect a significant effect of playback.

**Figure 6-8** The effects of early-life stress on the volume of the song-control nuclei HVC and RA in adult female song sparrows. RA = robust nucleus of the arcopallium, CORT = corticosterone. Data represent means ± SEM.
The effects of early-life stress on the induction of the immediate early gene Zenk in female song sparrows exposed to either conspecific or heterospecific (Gambel’s white-crowned sparrow) song. Birds were exposed to one of three different treatments during development: (A) control conditions, (B) food restriction, or (C) treatment with corticosterone (CORT). Zenk was quantified in three brain regions: the caudomedial mesopallium (CMM), the dorsal caudomedial nidopallium (dNCM), and the ventral caudomedial nidopallium (vNCM). Data represent means ± SEM. *p<0.05.
6.4 Discussion

Female song sparrows do not sing, but do show a preference for particular features of song produced by males in laboratory studies (Searcy, 1984; Nowicki et al., 2002b). Moreover, at least some of these song preferences depend on learning early in life (Hernandez et al., 2009). As predicted, exposure to developmental food restriction or CORT treatment affected both the behavioural and neural responses of female song sparrows to song. First, in study 1, control females hopped more in response to high complexity song than to low complexity song, whereas food-restricted and CORT-treated females did not discriminate in this way. In study 2, control subjects were more selective in their behavioural response to conspecific versus heterospecific song than food-restricted or CORT-treated subjects. Contrary to my predictions, I observed no long-term effects of either stressor on the volume of HVC or RA. However, early-life stress did affect Zenk induction in the adult auditory forebrain. Specifically, control females exposed to conspecific song showed higher Zenk induction in auditory forebrain regions than did control females exposed to heterospecific song, whereas Zenk induction in stressed females (food-restricted or CORT) was similar whether the females were exposed to conspecific or heterospecific song. Collectively these findings suggest that the altered behavioural response of food-restricted and CORT-treated females to song may be mediated by changes in the neural responsiveness of auditory forebrain regions to song presentation.

For the majority of the statistical comparisons in the present study, my sample sizes consisted of 7 or 8 individuals per group. This was the case for examining the effect
of the treatments on the behavioural response to high complexity versus low complexity
song and conspecific versus heterospecific song, since I used a repeated measures design
for these experiments. In addition, I also used sample sizes of 7 or 8 individuals per
treatment group for comparing the main effect of the treatments on the volume of the
song-control nuclei and the induction of Zenk in the auditory regions. These sample sizes
are typical of studies examining the brain and behaviour of wild birds, especially those
using hand-reared individuals (e.g. Nowicki et al., 2002a; Brenowitz et al., 1991). Sample
sizes were lower when examining the interaction between treatment and song type on
Zenk induction (3-5 individuals exposed to conspecific and heterospecific song for each
treatment). However, despite the small sample size for this analysis, the interaction term
was still significant (Fig 6-9). Therefore, for most of my comparisons, I am confident
that lack of statistical power was likely not the source of a lack of statistical significance
for measures where I did not observe group differences. However, to explore the
interaction between treatment and song type on Zenk induction, I compared Zenk levels
in individuals exposed to conspecific versus heterospecific song for each treatment group
separately. For these comparisons sample sizes were low (Table 6-1) and I cannot rule
out the possibility that I would have detected a significant difference for food-restricted
and CORT-treated individuals if sample sizes had been larger, although no trends in the
data were evident (Fig 6-9).
6.4.1 Activity levels as a measure of the behavioural response to song

One limitation of the current study is that few females performed copulation solicitation displays. This is a commonly used measure of song preference and discrimination in female songbirds (Searcy and Marler, 1981; Searcy and Capp, 1997; MacDougall-Shackleton et al., 1998a). However, most studies using this behavioural assay capture adult birds or use domesticated strains such as zebra finches or canaries where young are raised in colonies and exposed to adults. In the current study, females were reared in captivity from just over 3 days of age, which may have affected development of normal sexual behaviour. Therefore, I also used activity levels (number of hops) as a measure of the selectivity and strength of their behavioural response to song. The number of hops has been widely used as a dependent measure of female preference for song in a variety of songbird species (Bennett et al., 1996; Evans et al., 2006; Woodgate et al., 2010; Woodgate et al., 2011). In these previous studies, females were presented with multiple males or song stimuli simultaneously and the number of hops made on the perch in front of each potential choice was recorded, thus recording the number of visits to each male or song stimuli. In the present study, song stimuli were not presented simultaneously, thus I recorded activity levels in response to the different song stimuli. Similarly, a previous study in female mountain white-crowned sparrows (*Zonotrichia leucophrys oriantha*) used locomotor activity (number of hops) as a measure of preference for male song of natal dialect over male song of a foreign dialect (Baker et al., 1982). In that study, females performed both more copulation solicitation displays and more hops in response to songs from their natal dialect versus the foreign dialect,
suggesting that birds that perform more copulation displays exhibit higher levels of activity. Similarly, for birds in my study that did display, I found the number of copulation displays to be positively related to the number of hops. Therefore, one possibility is that control females were more active in response to conspecific song than to heterospecific song because they preferred conspecific song. However, in another study of white-crowned sparrows the number of hops was negatively correlated to the number of copulation displays (Maney and Wingfield, 1998), suggesting that activity levels are not always indicative of preference. Therefore, although I can conclude that control females were more selective in their behavioural response to conspecific song, I cannot be sure that this greater selectivity in response was indicative of a stronger preference per se.

6.4.2 Response to high complexity song

In study 1, control subjects hopped more in response to high complexity than to low complexity song bouts. I also observed a trend, albeit not statistically significant, for females exposed to food restriction during development to hop more to high complexity song bouts. This finding replicates previous reports that female song sparrows show a stronger behavioural (sexual) response to song bouts that contain more song types (Searcy, 1984). Consistent with this, females that did perform copulation solicitation displays performed more displays in response to high complexity than to low complexity song bouts. Therefore, female song sparrows appear to prefer high complexity song bouts, at least in an experimental setting. This preference appears to affect social mate
choice in the wild in some populations of song sparrow (e.g. Reid et al., 2004), although not others (e.g. Searcy, 1984).

Although only control females had a significantly stronger behavioural response to high complexity song, I observed no significant difference between the treatment groups in behavioural response scores. Similarly, female zebra finches exposed to early-life food restriction did not differ in the strength or direction of their preference for high complexity song compared to birds raised in control conditions (Woodgate et al., 2011). These findings suggest that developmental stressors may have only subtle effects on the behavioural response of females to songs that vary in complexity. Indeed, although it has been demonstrated that preferences for geographically local song are learned early in life (Hernandez et al. 2009), the precise mechanisms and timing for the development of preference for complex song remain unclear. It is possible that such preferences may depend on cognitive functions (such as habituation reduction; Searcy, 1992) that are relatively robust to developmental stressors. In my study, mean behavioural response scores of control females were close to 0.60. Although this was significantly above 0.50 (no selectivity in response), it may not be a strong enough response to readily detect a significant difference from individuals that do not show selectivity in their behavioural response for either song bout. Thus teasing apart whether very strong preferences for song complexity are affected by developmental stress will require further study.

6.4.3 Response to conspecific song

Female song sparrows raised under control conditions showed a significantly stronger behavioural response to conspecific versus heterospecific (white-crowned
sparrow) song. This finding is consistent with several previous findings that female songbirds generally prefer their own species’ song. For example, female song sparrows perform more copulation solicitation displays to song sparrow song than to swamp sparrow or chaffinch (Fringilla coelebs) song (Searcy and Marler, 1981). Female zebra finches also perform more copulation displays to conspecific song than heterospecific song (MacDougall-Shackleton et al., 1998a) and hop more on a perch that triggers zebra finch song versus a perch that triggers European starling song (Braaten and Reynolds, 1999). In contrast to subjects raised in control conditions, females exposed to food restriction or CORT treatment during development did not show a significantly stronger behavioural response to conspecific song. In addition, the strength of the selectivity of their behavioural response to conspecific song was significantly weaker among females exposed to early-life stress than among females reared in control conditions.

There are two possible explanations for this difference in behaviour. First, females exposed to developmental food restriction or CORT treatment may be unable to discriminate between the songs of the two species. Alternatively, they may be less motivated to participate in the behavioural testing trials and perhaps to attend to songs produced by potential mates. Consistent with this latter explanation, female zebra finches raised in experimentally enlarged broods were 3 times less active during mate choice trials than were females raised in small broods, suggesting that they were less motivated to seek a mate (Woodgate et al., 2010). Birdsong is a sexually selected trait in many species, and multiple aspects of song can correlate with measures of physiological condition and reproductive success (Kipper et al., 2006; Pfaff et al., 2007). If females exposed to early-life food restriction or CORT treatment are less motivated to attend to
songs produced by males, they may be less choosy when selecting a mate. As a result, they could mate with males that are of poorer phenotypic quality, which could negatively affect a female’s reproductive success.

6.4.4 Song control system

I detected no effect of early-life food restriction or CORT treatment on the volume of either RA or HVC in adult females. This is in contrast to previous studies that have found effects of early-life stress on the volumes of the song-control nuclei in males. For example, early food restriction and CORT treatment decrease the size of HVC, but not RA, in adult male zebra finches (Buchanan et al., 2004). In male swamp sparrows, food restriction decreased the absolute size of HVC and RA; the telencephalon was also smaller in food-restricted birds, resulting in the relative volume of RA (RA/telencephalon), but not HVC, being smaller in food-restricted subjects (Nowicki et al., 2002a). I found that food-restriction decreased the size of RA in male song sparrows (Chapter 5, Fig 5-5). Only one previous study has looked at the effects of early-life stress on volume of the song-control nuclei in females. In this study, both female and male song sparrows had a smaller HVC (but not RA) volume than birds raised in control conditions (MacDonald et al., 2006). However, in this study volume estimations were made in 23 day old birds immediately following experimental manipulation, whereas in my study birds were ~9 months old and were about 7 months post-manipulation. Since the brain of adult song sparrows maintains a large amount of plasticity (Soma et al., 2004; Newman et al., 2010), it is possible that the effects of stress on the song-control nuclei in female song sparrows disappear once the stressor subsides. Since I found no long-term effect on the
volume of HVC or RA, the changes in behaviour in response to song that were observed in the current study may not be mediated by alterations in the song control nuclei. However, I cannot exclude the possibility that song-control nuclei could differ between treatment groups in ways that were not examined, such as the density or size of neurons, or in their connectivity and function.

6.4.5 Zenk induction in auditory areas

Previous work has shown that levels of the immediate early gene Zenk are increased in NCM and CMM in response to conspecific song. For example, zebra finches and canaries exposed to conspecific song have higher Zenk induction compared to birds exposed to heterospecific song, a pure tone, or silence (Mello and Clayton, 1994). In addition, Zenk induction in these regions is increased in response to sexually relevant variation in song, such as the dialect (Maney et al., 2003) or length (Gentner et al., 2000) of songs. In the current study, consistent with previous findings, control females exposed to conspecific song had higher Zenk induction than did control females exposed to heterospecific song. However, food-restricted and CORT-treated females exposed to conspecific song had similar Zenk induction as individuals exposed to heterospecific song. Therefore, individuals exposed to early-life food restriction or CORT treatment were less selective in their behavioural response to conspecific versus heterospecific song and also failed to show the typical increase in Zenk levels in auditory forebrain regions in response to conspecific song. However, white-crowned sparrow song is acoustically similar to song sparrow song and it is possible that CORT-treated and food-restricted
birds would have exhibited behavioural and neural differences to a heterospecific song that was more dissimilar to song sparrow song (e.g. zebra finch).

The effect of stress on Zenk induction in the brain was observed 7 months after the cessation of the stress treatments. Therefore, early-life food restriction and CORT treatment may have persistent effects on neural responses in song sparrows long after the stressor subsides. In zebra finches, lesions of CMM prevent accurate discrimination of conspecific song over heterospecific song (MacDougall-Shackleton et al., 1998a), suggesting that the auditory forebrain regions of the songbird brain have an important role in the perception of song features that are important for female song preferences. Therefore, it is possible that early-life food restriction and CORT treatment impaired development of NCM or CMM in female song sparrows and that this is at least partially responsible for the alterations in the behavioural response to conspecific song. There are multiple reasons why Zenk induction in response to conspecific song may be lower in females exposed to early-life stress. First, the density of neurons may be decreased in females exposed to stress. This could occur if stress decreased neurogenesis during development. Consistent with this, a variety of stressors decrease neurogenesis in developing rats (Gould and Tanapat, 1999) and chronic administration of CORT reduces cell proliferation in the brain of adult song sparrows (Newman et al., 2010). Alternatively, instead of affecting the number of neurons, the stressors could permanently alter the function of neurons in the brain. To date no studies have examined the effects of early-life stress on neuronal function in adult songbirds, but rats exposed to chronic stress during development exhibit alterations in long-term-potentiation and dendritic morphology of hippocampal neurons in adulthood (Brunson et al., 2005).
In Chapter 2, I determined the effects of developmental food restriction or CORT treatment on nestling growth rates and adult metabolic rates, body size, and body composition. In that Chapter, I found that both food restriction and CORT treatment decreased growth in females and increased standard metabolic rates in adulthood, but there was no long-term effect of either treatment on adult body size, body composition (lean or fat mass), or peak metabolic rates (Schmidt et al., 2012a). Collectively, these findings suggest that developmental stressors that are not severe enough to have detectable long-term effects on many morphological and physiological traits may still have persistent effects on the brain and behaviour. Thus, in songbirds, the brain may be particularly susceptible to the effects of early-life stressors.

6.4.6 Conclusions

Female song sparrows exposed to food restriction or CORT treatment during development were less selective in their behavioural response to conspecific versus heterospecific song than females reared in control conditions. Female song sparrows exposed to developmental food restriction or CORT treatment may be less able to discriminate between songs of varying quality or be less motivated to attend to songs of potential mates, which could affect their eventual choice of mates in the wild. This effect on behaviour was paralleled by alterations in Zenk induction in the auditory forebrain regions in response to song presentation. These results show that stressors known to affect male song production might also affect neural processing of song by females, and the strength of their subsequent song preferences.
References


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Chapter 7

7 General Discussion

7.1 Summary

In this thesis, I provided a rigorous test of the Developmental Stress Hypothesis. In Chapters 2-4, I determined the effects of early-life food restriction or corticosterone (CORT) treatment on several physiological traits related to adult phenotypic quality. In Chapter 2, I found that although early-life food restriction and CORT treatment affected nestling growth, there was no effect of either stressor on adult body size, body composition, or peak metabolic rates in males or females. Standard metabolic rate (SMR) was elevated in females exposed to early-life stress, but not males. In Chapter 3, I found that both stressors suppressed swelling of the wing-web in response to phytohemagglutinin (PHA) in males, but not females. Early-life CORT treatment also decreased the ability of males to eliminate a strain of *E. coli* bacteria, but increased their ability to eliminate a strain of *C. albicans* fungus. In Chapter 4, I found that both males and females exposed to CORT treatment during development had exaggerated increases in CORT in response to a standardized dose of adrenocorticotropic hormone (ACTH) in adulthood. Males treated with CORT during development also had higher initial (unchallenged) levels of circulating testosterone, whereas females exposed to either stressor had lower levels of 17β-estradiol.
In Chapters 5 and 6, I determined the effects of early-life food restriction or CORT treatment on the brain and behaviour. Specifically, in Chapter 5, I showed that early-life food restriction and CORT treatment affect the quality of adult male song production. Males exposed to either stressor sang less complex song in adulthood, and food-restriction decreased the accuracy of song learning and impaired development of the robust nucleus of the arcopallium (RA). Lastly, in Chapter 6, I showed that early-life food restriction and CORT treatment also affect the brain and behaviour of female songbirds. Females exposed to either treatment were less selective in their behavioural and neural response (as indicated by Zenk induction) to conspecific versus heterospecific song.

Many features of this thesis are novel. First, this is the first study to examine the effects of early-life stress on song production and a variety of physiological traits in the same individuals. This allowed me to determine if the same stressors that affect adult male song production also affect adult phenotypic quality, which is a key prediction of the Developmental Stress Hypothesis. Second, this is one of the first studies to determine the effects of early-life stress on male song-repertoire size, since most studies to date have been conducted in species that sing only one or a few unique song types (Nowicki et al., 2002; Spencer et al., 2003). Third, this is also the first study to determine the effects of developmental stress on measures of vocal performance. Fourth, this is one of few studies to determine the relationship between paternal song quality and offspring song quality to determine which features of song may be influenced by hereditary factors. Fifth, this is one of the few studies to determine the effects of early-life stress on the behaviour of female songbirds, and the first study to examine the long-term effects of
early-life stress on the brain of female songbirds. Indeed, this is the first study to
determine the long-term effects of chronic early-life stress on induction of an immediate
early gene in the brain of any species.

7.2 Developmental programming

The findings of this thesis add to a growing body of evidence showing that
variation in the early rearing environment can have long-term effects on adult phenotype.
This process is often referred to as developmental programming, whereby environmental
stimuli, especially exposure to stressors, induce widespread physiological changes to
many developing organs and systems in the body (McMillen and Robinson, 2005). These
changes may exist long-term or may even be permanent. In the short term, physiological
changes in response to stressors may be beneficial (referred to as immediate adaptive
responses, Gluckman et al., 2005b), for example by changing patterns of somatic growth
to increase the chance of short-term survival. In western spadefoot toads (Scaphiopus
hammondii,) experimentally simulating pond desiccation causes tadpoles to accelerate
maturation and complete metamorphosis at a younger age (Denver et al., 1998), which
allows them to escape the deteriorating conditions. Similarly, I found that male song
sparrow nestlings treated with CORT during development weighed more than controls. In
the wild, accelerated growth may allow nestlings to fledge the nest early in order to
escape a stressful environment.

Although potentially beneficial in the short term, the physiological changes
induced by early-life stressors may negatively affect health and fitness in the long-term.
This observation gave rise to the developmental origins of adult disease paradigm,
whereby the responses to environmental stressors that may be beneficial in the short term are believed to increase the risk of adulthood diseases (Gluckman et al., 2005a; Gluckman et al., 2005c). For example, many studies in humans have found that intrauterine growth restriction, as evidenced by low birth weight, is associated with an increased risk of obesity, diabetes, and cardiovascular disease in adulthood (Gluckman et al., 2005a; McMillen and Robinson, 2005). In addition, to metabolic disorders, early-life stressors also affect brain development in humans and can impair cognition and increase the risk of mental illness later in life (de Kloet et al., 2005; Welberg and Seckl, 2001).

Similarly, in this thesis I found that exposure to developmental food restriction or CORT treatment had long-term effects on the brain and behaviour of male and female song sparrows. In addition, my manipulations also had long-term, programming effects on the hypothalamic-pituitary-adrenal (HPA) axis, hypothalamic-pituitary-gonadal (HPG) axis, immune function (males only), and standard metabolic rates (females only).

An important point to consider is that the vast majority of studies that examine the long-term effects of early-life stress assess adult phenotype under ideal environmental conditions. That is, individuals are typically exposed to stressors for a short period of time during development and then the stressor is terminated and individuals live in standard laboratory conditions until adult phenotype is assessed. However, recent hypotheses propose that the specific effects of early-life stress may depend on the quality of the adult environment (Sheriff and Love, 2013). Specifically, the Environmental Matching Hypothesis proposes that the negative effects of early-life stress that are commonly observed when adult individuals are tested in ideal environmental conditions may disappear, or even be reversed, if individuals are tested in a low quality environment.
(Monaghan, 2008). Under this hypothesis, the physiological changes that occur in response to developmental stressors are thought to prepare the young organism to live in a harsh environment. However, if the environment changes such that conditions are no longer harsh (an environmental mismatch), these individuals will be at a disadvantage compared to individuals that grew up in a high quality environment. To date very few studies have tested this hypothesis. In one experiment, male nestling zebra finches reared in an experimentally enlarged brood had suppressed constitutive innate immune function in adulthood, however this effect went away when individuals were assessed under unfavourable adult conditions (De Coster et al., 2011). In this thesis, CORT-treated males exhibited accelerated growth, low complexity song, suppressed immune function, heightened CORT responses to ACTH, and higher initial testosterone levels. Some of these changes may appear detrimental, but perhaps in a harsh environment it would be beneficial to tradeoff development of the immune system and brain in favour of growth to ensure survival in the short-term. In addition, if conditions were poor and life expectancy short then investment in immune function may be less important. Also, a hyperactive HPA axis may cause individuals to be more vigilant in a harsh environment (Sheriff and Love, 2013) and high testosterone levels may cause them to be more focused on reproduction and securing a mate (Wingfield et al., 1990), which may be beneficial if living to another breeding season is unlikely. However, this is all speculative and future studies are required to determine how variation in the quality of the adult environment is related to the effects of early-life stress on health and fitness.
7.3 Mechanisms by which stress affects development

Many different types of environmental stressors can have the same effect on development. For example, food restriction (Spencer et al., 2003), CORT treatment (Spencer et al., 2003), parasite exposure (Spencer et al., 2005), and increased sibling competition (Brumm et al., 2009) have all affected song learning in songbirds, in at least some studies. The mechanisms by which these varied stressors all affect development is unclear, but one hypothesis is that they all increase levels of CORT, which then affects development of the song-control system (Spencer et al., 2003). Similarly, increased CORT may also affect development of the HPA axis (Spencer et al., 2009), immune function (Rubolini et al., 2005), and body size in some species (Spencer et al., 2003). If this hypothesis were correct, than these stressors should increase CORT levels. Indeed, studies in birds have found that food restriction can increase both baseline (Kempster et al., 2007) and stress-induced CORT levels (Kitaysky et al., 2001). However, in this thesis food restriction did not elevate circulating CORT levels at either 10 or 45 days of age, which may suggest that increased CORT was not the mechanism that mediated the effects of food restriction on development. However, I cannot rule out the possibility that CORT was involved. For example, it is possible that food-restriction elevated CORT levels during a specific time in the treatment period when blood samples were not collected. In addition, food-restriction may have elevated stress-induced CORT levels, as opposed to baseline (Buchanan et al., 2003), which were not measured in this study. Lastly, there are many other factors that can influence the exposure of tissues to CORT that could have been affected by food restriction, such as the expression of corticosteroid receptors or enzymes that locally metabolize CORT in tissues (Schmidt et al., 2008).
If increased CORT mediates the effects of environmental stressors on development, than another prediction is that CORT and environmental stressors should have similar effects on adult phenotype. Consistent with this, in zebra finches, both food restriction and CORT treatment decrease nestling growth, impair development of HVC, and decrease the quality of adult song production (Buchanan et al., 2004; Spencer et al., 2003). Similarly, in this thesis I found that both food restriction and CORT treatment affected the behavioural response of females to conspecific song and the induction of the immediate early gene Zenk in the auditory forebrain following song presentation. CORT and food restriction also had similar effects on song complexity in males. In addition, both stressors had similar effects on nestling growth and adult standard metabolic rate (SMR) in females and on the swelling response to PHA in males. This suggests that CORT may mediate the effects of food restriction on these traits. However, only food restriction affected the volume of RA and song learning accuracy in males, suggesting that CORT may not be the only mechanism mediating the effects of food restriction on the brain and behaviour. Future studies that more directly determine the role of CORT in mediating the effects of environmental stressors would be valuable, for example, by administering a CORT synthesis inhibitor to food restricted subjects to see if the effects of food restriction on brain development and song production are no longer evident.

7.4 Song as an indicator of developmental stress

The findings in this thesis support the Developmental Stress Hypothesis: early-life stress had long-term effects on the quality of adult male song production. Specifically, I found that both song complexity and song learning accuracy were affected by
developmental food restriction and CORT treatment, suggesting that these features of song are honest indicators of a male’s early ontogeny. Importantly, not all measures of song were affected by my experimental manipulations and my results suggest that early-life food restriction or CORT treatment do not affect vocal performance in song sparrows. Interestingly, I also found that paternal repertoire size was significantly related to offspring vocal performance (trill deviation). This relationship could be due to hereditary factors, or possibly maternal effects. Thus the quality of adult song production is likely determined by both a male’s genotype and the quality of his early-rearing environment. Although I found that early-life stress affected the quality of adult song production, it is unclear if the differences I observed between treatment groups would affect a male’s ability to interact with conspecifics. Thus, future studies that determine if the songs from males exposed to early-life stress are less effective in stimulating females or in eliciting aggressive responses from males would be beneficial.

I also found that early-life stress affected multiple physiological traits in song sparrows. Therefore, in addition to singing a poorer quality song, adult males exposed to developmental stress had altered immune function, higher circulating testosterone levels, and larger increases in CORT in response to ACTH. Since variation in these physiological traits is related to health and fitness (Breuner et al., 2008; Moller and Saino, 2004; Silverin, 1980) this could have important consequences for the direct benefits received by female mates. A male with suppressed immune function may be more likely to get sick and thus less able to secure a high quality territory or help care for young. In addition, a male with a hyperactive HPA axis may be more inclined to abandon a nest and may provide less parental care (Silverin, 1986). Similarly, if testosterone levels
remained high throughout the breeding season males may sacrifice parental care in favour of mate guarding and territory defense (Hegner and Wingfield, 1987; Silverin, 1980). Therefore, by choosing a mate based on song production a female may gain access to a male who experienced less stress during development and consequently a higher quality mate, which may increase her own fitness. In addition to birdsong, other sexually selected traits may serve as indicators of development (Spencer and MacDougall-Shackleton, 2011). For example, both fluctuating asymmetry of bilateral traits (Moller, 1992) and melanin-based plumage colouration (Roulin, 1999) appear to be sexually selected traits in some species. Furthermore, expression of both these traits may be affected by exposure to developmental stressors (Parsons, 1990; Roulin et al., 2008) and related to measures of adult phenotypic quality (Furlow et al., 1997; Roulin et al., 2001; Thornhill and Gangestad, 2006). Thus, birdsong may be just one example of a general process whereby indicators of development come to be sexually selected traits (Spencer and MacDougall-Shackleton, 2011).

7.5 Conclusions

In conclusion, I used an integrative and interdisciplinary approach to determine the effects of early-life stress on the behaviour, neuroanatomy, and physiology of song sparrows. Results from the studies outlined in this thesis contribute many novel findings to the fields of behavioural ecology, physiology, and neuroscience. I showed that early-life food restriction and CORT treatment had long-term effects on several aspects of adult phenotype, which illustrates the importance of considering developmental conditions when trying to determine the factors responsible for inter-individual variation in adult
trait expression. In addition, by analyzing the relationship between the experimental subjects and their genetic fathers I was also able to show the importance of hereditary factors in some traits. My results suggest that birdsong is an honest indicator of a male’s early developmental environment and subsequent adult phenotype. Thus a female that chooses a male based on the quality of song production could gain access to a higher quality male which may provide her with direct fitness benefits.
7.6 References


Hypothesis": theoretical implications for patterns of testosterone secretion, mating
Appendix 1 Relationship between Song Production and Phenotypic Quality in Adult Males Song Sparrows

Introduction

In many species of songbirds, song is considered a sexually selected trait with females showing a preference for a variety of attributes of song production. For example, females may prefer males with more complex song repertoires (Searcy, 1984), that produce physically challenging notes such as trills (Ballentine et al., 2004), that sing longer song bouts (Gentner and Hulse, 2000), or that produce songs from the female’s natal dialect (MacDougall-Shackleton et al., 2001). This has led to the hypothesis that song may be an honest indicator of male quality. Thus, females who choose a mate on the basis of song production could gain access to a higher quality mate. This may in turn provide her with direct benefits (e.g. increased parental care), indirect benefits (e.g. good genes being passed on to her offspring), or both. In support of this, past studies have found that many song features predict male phenotypic quality and fitness. For example, in free-living song sparrows (*Melospiza melodia*), song complexity is correlated with regulation of the hypothalamic-pituitary-adrenal (HPA) axis (MacDougall-Shackleton et al., 2009; Schmidt et al., 2012a), body condition (Pfaff et al., 2007), immune function (Reid et al., 2005a), and ability to defend a territory (Hiebert et al., 1989). Furthermore, in one study, male song sparrows with larger song repertoires contributed more offspring and grand offspring to the population because they lived longer and raised a larger number of nestlings to independence (Reid et al., 2005b).
Current sexual selection theories posit that in order for a trait to be an honest indicator of the quality of the signaler, there should be some cost associated with the production or maintenance of the trait (Zahavi, 1975). Otherwise, the signaler could benefit from exaggerating the trait to appear of higher quality than they actually are, which would negate any benefits to the receiver (Zahavi, 1975). It has been difficult to determine the costs of certain song attributes, such as song complexity or singing specific geographic song variants. However, a relatively recent hypothesis, The Developmental Stress Hypothesis, was proposed to explain the costs of these song features and has begun to accumulate a great deal of empirical support (Nowicki et al., 1998; Nowicki et al., 2002). The Developmental Stress Hypothesis proposes that the costs of some song features are associated with the development of the neural structures that are important for the learning and production of song (the song-control system). The song-control system develops during the nestling and fledgling period in songbirds and during this time young birds are likely to be exposed to a variety of environmental stressors, especially food restriction since nestlings are dependent on their parents for food. This hypothesis proposes that males who experience fewer stressors will experience superior neural development and consequently will produce the best quality song in adulthood. Thus, some song features may be an honest indicator of a male’s early ontogeny and subsequent adult phenotype (Nowicki et al., 2002). A key prediction of this hypothesis is that early-life stress should affect development of the song-control system and adult song production. Many experimental studies have now found support for this prediction (reviewed in MacDougall-Shackleton and Spencer, 2012).
Another assumption of the Developmental Stress Hypothesis is that early-life stress should have long-term effects on a variety of other traits that could be important for the fitness of potential mates. (Nowicki et al., 2002) That is, males exposed to developmental stress should be of lower phenotypic quality in adulthood, which would affect the direct benefits received by their mates. There is some support for this prediction. For example, European starlings (*Sturnus vulgaris*) exposed to food restriction during development performed worse on a spatial foraging task in adulthood (Farrell et al., 2011). In zebra finches, treating nestlings with the glucocorticoid hormone corticosterone (CORT) permanently affects the hypothalamic-pituitary-adrenal (HPA) axis (Spencer et al., 2009). I recently found more support for this prediction in song sparrows. Specifically, I found that early-life stress had long-term effects on immune function (Chapter 3), the HPA axis (Chapter 4), and the hypothalamic-pituitary-gonadal (HPG) axis (Chapter 4). However, not all traits appear to be affected by early-life stress, as I found no long-term effect of stress on body size, body composition, or metabolic rates in male song sparrows (Chapter 2; Schmidt et al., 2012b).

If song is an honest indicator of a male’s early ontogeny, then another logical prediction of the Developmental Stress Hypothesis is that song should be correlated with measures of adult phenotypic quality in individuals exposed to early-life stress. That is, early-life stress should affect adult song production and male phenotypic quality in the same individuals, such that song is correlated to these other traits in adulthood. Although a variety of studies have confirmed the effects of developmental stress on adult song production and a few other behavioural and physiological traits, it is unclear if song and these other traits are correlated in individuals exposed to early-life stress. To date, only
one study has tested this prediction. In this study, exposing European starlings to food restriction early in life resulted in males that sang shorter song bouts and that performed worse on a spatial foraging task (Farrell et al., 2011). In addition, song bout length was significantly correlated with performance on the spatial task, such that males with longer song bouts tended to perform fewer errors while completing the task (Farrell et al., 2011). This suggests that song and spatial learning may be developmentally correlated traits in European starlings. Here I use the term developmentally correlated traits to refer to two functionally independent traits that are correlated in adulthood because of the organizational effects of early-life stress (Spencer and MacDougall-Shackleton, 2011).

In the current study, I determined the relationship between 5 attributes of adult song production (syllable repertoire size, song-type repertoire size, song learning accuracy, song stereotypy, trill deviation) and several measures of adult phenotype in male song sparrows that were raised under control conditions or exposed to early-life stressors (food restriction or CORT treatment). My measures of adult phenotype included several physiological, morphological, and neuroanatomical traits including: 1) neuroanatomy of the song-control system, 2) body size, body composition, and metabolic rates, 3) innate and acquired immune function, 4) regulation of the HPA axis, and 5) regulation of the HPG axis. In the previous chapters of this thesis I showed that early-life stress affects adult song production and some of these measures of adult phenotype. However, in this chapter I determined if song is significantly related to these traits. Song may be correlated to these traits because of the organizational effects of early-life stress (developmentally correlated traits). Alternatively, song could be correlated to other traits
even if neither trait was affected by early-life stress because of genetic factors or because of individual variation in current condition, as opposed to developmental conditions.

Methods

Subjects

Male subjects used in the current study were the same as those used in Chapter 2. (Schmidt et al., 2012b) and sample sizes were identical (control: n=9, food-restricted: n=8, CORT-treated: n=6).

Song measurements

Song repertoires were recorded from May to July 2011 when birds were ~1 year of age. Details of the recording procedure and song analyses are described in Chapter 5 (Schmidt et al., 2013). I measured 5 song attributes: syllable repertoire size, song-type repertoire size, song learning accuracy, song stereotypy, and trill deviation.

Measures of phenotypic quality

The song-control system

As an indicator of brain development, I measured the volume of three brain regions that comprise the song-control system: HVC, the robust nucleus of the arcopallium (RA) and area X. These brain regions are important for both the learning and production of song (Scharff and Nottebohm, 1991). In addition, I also measured the number of NeuN+ cells in HVC (as an indicator of neuron number) because previous
studies have found that CORT affects the number of proliferating cells and the total number of neurons in this region in adult song sparrows (Newman et al., 2010). Details of the neuroanatomical procedures are described in Chapter 5 (Schmidt et al., 2013).

Body size, body composition, and metabolic rates

Measurements of body size, body composition, and metabolic rates were completed from December 2010 to January 2011 when birds were ~7 months old. Details of the procedures for all measurements can be found in Chapter 2 (Schmidt et al., 2012b). For body size, I used the principal component analysis (PCA) scores to represent one overall measure of body size. Morphological measures included in the PCA were body mass, wing chord, and tarsus. Measures of lean mass, fat mass, standard metabolic rate (SMR), peak metabolic rate (PMR), and metabolic scope (PMR/SMR) were also included in the analyses. Lastly, I measured body mass at the time that birds were euthanized when they were ~1 year old and this measure was also included in the statistical analyses.

Immune function

I took several measurements of innate and acquired immune function from September to October 2010 when subjects were ~5 months old. Details of these measurements are described in Chapter 3. Measures of immune function included in the current study include swelling of the wing web in response to phytohemagglutinin (PHA) and leukocyte profiles (the number of lymphocytes, number of heterophils, heterophil:lymphocyte [H:L] ratios). In addition, I included the three PCA scores for the six measures of constitutive innate immune function as described in Chapter 3. The measures of constitutive innate immune function included in the PCA were microbicidal
activity against the three microbes (*E. coli* #8739, *E. coli* #51813, *C. albicans*), lysis and agglutination scores from the hemolysis-hemagglutination assay, and plasma lysozyme concentrations.

*HPA axis regulation*

I measured 3 components of HPA axis regulation in April and May 2011 when birds were ~11.5 months old, as described in Chapter 4. This included the response of plasma CORT to an acute restraint stressor, efficacy of negative feedback of the HPA axis by determining the ability of the exogenous glucocorticoid dexamethasone (DEX) to suppress endogenous CORT levels, and adrenal sensitivity to an injection of exogenous adrenocorticotropic hormone (ACTH).

*HPG axis regulation*

I assessed functioning of the HPG axis in April to May 2011 when birds were ~11.5 months old, as described in Chapter 4. This included a measure of initial (unchallenged) testosterone levels, as well as testosterone levels 30 and 60 min after a gonadotropin releasing hormone (GnRH) challenge.

**Data analysis**

All statistical analyses were performed in SPSS version 20 (IBM, Armonk, NY, USA). I conducted a correlation matrix to determine if the 5 song attributes (syllable repertoire, song-type repertoire, learning accuracy, song stereotypy, trill deviation) were correlated with the several measures of phenotypic quality. This also allowed me to examine the relationship between the individual measures of phenotypic quality (see
Table A-5 at the end of this appendix) as well as the relationships between the multiple measures of song production.

In addition, I also used step-wise multiple linear regressions to determine if the measures of song production were related to the measures of adult phenotypic quality. This allowed me to look at the relationship between song and each individual measure of phenotypic quality, while controlling for variation in all of the other traits. I performed 5 regressions in total, with each one of the 5 song measures entered as a dependent variable. The measures of phenotypic quality were then included as independent variables. I report the results from both the correlation matrix and the multiple regression analyses and in most cases the results were the same for the two analyses. Statistical significance was set at $\alpha=0.05$ and all tests were two-tailed.

Results

Syllable repertoire size

Results from the correlation matrix analysis indicated that syllable repertoire size was positively correlated to song-type repertoire size ($r=0.898$, $p<0.001$) and song learning accuracy ($r=0.455$, $p=0.034$). Syllable repertoire size was also positively correlated with the volume of RA ($r=0.503$, $p=0.017$). In addition, syllable repertoire size was correlated with two physiological traits, including a positive correlation with the swelling response to PHA ($r=0.431$, $p=0.045$). This suggests that birds with more syllables in their repertoires had a stronger immune response to PHA. Syllable repertoire
size was positively correlated with the response of CORT to DEX \( (r=0.467, p=0.029) \). Since the response to DEX was calculated as CORT levels post-injection of DEX - CORT levels post-restraint, this indicates that subjects that were less able to suppress CORT in response to DEX, and who thus had less effective negative feedback, had more syllables in their repertoires. The multiple regression analysis \( (r^2=0.626, F_{3,15}=7.803, p=0.003) \) showed similar relationships to the measures of phenotypic quality (Table A-1).

Again, syllable repertoire size was significantly and positively related to the volume of RA \( (\beta=0.527, t=3.221, p=0.006) \) and the swelling response to PHA \( (\beta=0.356, t=2.151, p=0.049) \). The relationship between syllable repertoire size and the suppression of CORT by DEX was no longer significant. However, syllable repertoire size did have a significant negative relationship with plasma testosterone levels 30 min post-GnRH challenge \( (\beta=-0.396, t=-2.394, p=0.031) \).

**Song-type repertoire size**

Results from the correlation matrix indicated that song-type repertoire size was positively correlated with syllable repertoire size \( (r=0.898, p<0.001) \). The only measure of phenotypic quality that was significantly correlated to song-type repertoire size was the swelling response to PHA. Subjects with more song types in their repertoires had a stronger swelling response to PHA \( (r=0.0568, p=0.006) \). Results from the multiple regression analysis \( (r^2=0.320, F_{1,15}=8.988, p=0.009) \) were similar (Table A-2). The only measure of phenotypic quality significantly related to song-type repertoire size was the swelling response to PHA \( (\beta=0.600, t=2.998, p=0.009) \).
Table A- 1 Results from step-wise multiple regression analysis showing the relationship between syllable repertoire size and measures of adult phenotype in male song sparrows

<table>
<thead>
<tr>
<th></th>
<th>Beta</th>
<th>t statistic</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA Volume</td>
<td>0.527</td>
<td>3.221</td>
<td>0.006</td>
</tr>
<tr>
<td>T 30 min</td>
<td>-0.396</td>
<td>-2.394</td>
<td>0.031</td>
</tr>
<tr>
<td>PHA Swelling</td>
<td>0.356</td>
<td>2.151</td>
<td>0.049</td>
</tr>
<tr>
<td>PMR (W)</td>
<td>0.276</td>
<td>1.725</td>
<td>0.108</td>
</tr>
<tr>
<td>Metabolic Scope</td>
<td>0.244</td>
<td>1.407</td>
<td>0.183</td>
</tr>
<tr>
<td>Immune PC2</td>
<td>0.232</td>
<td>1.312</td>
<td>0.212</td>
</tr>
<tr>
<td>Immune PC3</td>
<td>0.210</td>
<td>1.289</td>
<td>0.220</td>
</tr>
<tr>
<td>CORT Dex</td>
<td>0.182</td>
<td>1.036</td>
<td>0.319</td>
</tr>
<tr>
<td>Lean mass (g)</td>
<td>0.183</td>
<td>0.949</td>
<td>0.360</td>
</tr>
<tr>
<td>CORT restraint</td>
<td>-0.149</td>
<td>-0.804</td>
<td>0.436</td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td>0.161</td>
<td>0.696</td>
<td>0.498</td>
</tr>
<tr>
<td>T 3 min</td>
<td>-0.117</td>
<td>-0.602</td>
<td>0.558</td>
</tr>
<tr>
<td>SMR (W)</td>
<td>0.136</td>
<td>0.577</td>
<td>0.574</td>
</tr>
<tr>
<td>Mass (g)</td>
<td>-0.135</td>
<td>-0.544</td>
<td>0.596</td>
</tr>
<tr>
<td>Area X volume</td>
<td>0.092</td>
<td>0.461</td>
<td>0.652</td>
</tr>
<tr>
<td>Immune PC1</td>
<td>0.091</td>
<td>0.443</td>
<td>0.665</td>
</tr>
<tr>
<td>H:L</td>
<td>0.064</td>
<td>0.366</td>
<td>0.720</td>
</tr>
<tr>
<td>HVC volume</td>
<td>-0.063</td>
<td>-0.348</td>
<td>0.734</td>
</tr>
<tr>
<td>Body Size PCA</td>
<td>-0.056</td>
<td>-0.276</td>
<td>0.787</td>
</tr>
<tr>
<td>T 60 min</td>
<td>-0.061</td>
<td>-0.268</td>
<td>0.793</td>
</tr>
<tr>
<td>Heterophil:RBC</td>
<td>0.048</td>
<td>0.261</td>
<td>0.798</td>
</tr>
<tr>
<td>CORT ACTH</td>
<td>-0.028</td>
<td>-0.160</td>
<td>0.876</td>
</tr>
<tr>
<td>HVC NeuN+ cells</td>
<td>-0.031</td>
<td>-0.159</td>
<td>0.876</td>
</tr>
<tr>
<td>Lymphocyte:RBC</td>
<td>0.024</td>
<td>0.125</td>
<td>0.903</td>
</tr>
</tbody>
</table>

Note: values in bold were significantly related to syllable repertoire size
Table A-2 Results from step-wise multiple regression analysis showing the relationship between song-type repertoire size and measures of adult phenotype in male song sparrows

<table>
<thead>
<tr>
<th></th>
<th>Beta</th>
<th>t statistic</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA Swelling</td>
<td>0.600</td>
<td>2.998</td>
<td>0.009</td>
</tr>
<tr>
<td>RA Volume</td>
<td>0.377</td>
<td>2.063</td>
<td>0.057</td>
</tr>
<tr>
<td>SMR (W)</td>
<td>0.338</td>
<td>1.794</td>
<td>0.093</td>
</tr>
<tr>
<td>T 30 min</td>
<td>-0.339</td>
<td>-1.786</td>
<td>0.094</td>
</tr>
<tr>
<td>CORT Dex</td>
<td>-0.293</td>
<td>-1.516</td>
<td>0.150</td>
</tr>
<tr>
<td>CORT restraint</td>
<td>0.274</td>
<td>1.408</td>
<td>0.180</td>
</tr>
<tr>
<td>Lean mass (g)</td>
<td>0.259</td>
<td>1.327</td>
<td>0.204</td>
</tr>
<tr>
<td>Immune PC1</td>
<td>0.291</td>
<td>1.314</td>
<td>0.209</td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td>0.259</td>
<td>1.311</td>
<td>0.210</td>
</tr>
<tr>
<td>Heterophil:RBC</td>
<td>0.246</td>
<td>1.252</td>
<td>0.230</td>
</tr>
<tr>
<td>H:L</td>
<td>0.223</td>
<td>1.119</td>
<td>0.281</td>
</tr>
<tr>
<td>T 3 min</td>
<td>-0.215</td>
<td>-1.040</td>
<td>0.315</td>
</tr>
<tr>
<td>Mass (g)</td>
<td>0.200</td>
<td>0.998</td>
<td>0.334</td>
</tr>
<tr>
<td>HVC volume</td>
<td>0.149</td>
<td>0.698</td>
<td>0.496</td>
</tr>
<tr>
<td>HVC NeuN+ cells</td>
<td>0.150</td>
<td>0.678</td>
<td>0.508</td>
</tr>
<tr>
<td>Lymphocyte:RBC</td>
<td>0.127</td>
<td>0.618</td>
<td>0.546</td>
</tr>
<tr>
<td>PMR (W)</td>
<td>0.127</td>
<td>0.598</td>
<td>0.559</td>
</tr>
<tr>
<td>Body Size PCA</td>
<td>0.095</td>
<td>0.460</td>
<td>0.652</td>
</tr>
<tr>
<td>Area X volume</td>
<td>-0.105</td>
<td>-0.451</td>
<td>0.659</td>
</tr>
<tr>
<td>T 60 min</td>
<td>-0.082</td>
<td>-0.400</td>
<td>0.695</td>
</tr>
<tr>
<td>Immune PC2</td>
<td>-0.056</td>
<td>-0.263</td>
<td>0.796</td>
</tr>
<tr>
<td>Immune PC3</td>
<td>-0.050</td>
<td>-0.243</td>
<td>0.811</td>
</tr>
<tr>
<td>Metabolic Scope</td>
<td>-0.029</td>
<td>-0.134</td>
<td>0.895</td>
</tr>
<tr>
<td>CORT ACTH</td>
<td>0.000</td>
<td>0.002</td>
<td>0.999</td>
</tr>
</tbody>
</table>

Note: value in bold was significantly related to song-type repertoire size.
Learning accuracy

Song learning accuracy was positively correlated to syllable repertoire size \((r=0.455, p=0.034)\). However, results from both the correlation matrix and the multiple regression analysis indicate that learning accuracy was not significantly related to any of the measures of phenotypic quality (data not shown).

Song stereotypy

Results from the correlation matrix showed that song stereotypy was negatively correlated to both the volume of HVC \((r=-0.447, p=0.037)\) and the number of neurons in HVC \((r=-0.482, p=0.023)\). Thus individuals with more stereotyped song had smaller HVC volumes and fewer neurons in HVC. Song stereotypy scores were also negatively correlated to immune PC2 scores. Immune PC2 was largely explained by variation in the lysis of rabbit erythrocytes and antimicrobial capacity of plasma toward one strain of \(E. \ coli\) (#8739). This indicates that subjects with more stereotyped song had lower lysis titers and were less able to eliminate one strain of \(E. \ coli\). Results from the multiple regression analysis \((r^2=0.514, F_{2,15}=7.933, p=0.004)\) were comparable (Table A-3). Again song stereotypy was significantly and negatively related to the volume of HVC \((\beta=-0.482, t=-2.648, p=0.018)\) and immune PC2 scores \((\beta=-0.604, t=-3.324, p=0.005)\).

Trill deviation

Results from the correlation matrix indicate that trill deviation was negatively correlated to the volume of HVC \((r=-0.483, p=0.027)\). This suggests that subjects that produced trills with less deviation from the upper performance limit, and thus higher
performing trills, had a larger HVC. Trill deviation was also negatively correlated to the swelling response to PHA ($r=-0.572, p=0.007$): subjects with higher performing trills had stronger swelling responses to PHA. The results from the multiple regression analysis ($r^2=0.361, F_{1,15}=8.470, p=0.011$; Table A-4) also showed a negative relationship between trill deviation and swelling response to PHA ($\beta=-0.601, t=-2.910, p=0.011$).

Table A-3 Results from step-wise multiple regression analysis showing the relationship between song stereotypy and measures of adult phenotype in male song sparrows

<table>
<thead>
<tr>
<th>Measure</th>
<th>Beta</th>
<th>t statistic</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune PC2</td>
<td>-0.604</td>
<td>-3.324</td>
<td>0.005</td>
</tr>
<tr>
<td>HVC volume</td>
<td>-0.482</td>
<td>-2.648</td>
<td>0.018</td>
</tr>
<tr>
<td>T 3 min</td>
<td>-0.268</td>
<td>-1.428</td>
<td>0.175</td>
</tr>
<tr>
<td>CORT restraint</td>
<td>0.261</td>
<td>1.416</td>
<td>0.179</td>
</tr>
<tr>
<td>Mass (g)</td>
<td>0.292</td>
<td>1.379</td>
<td>0.189</td>
</tr>
<tr>
<td>Lean mass (g)</td>
<td>0.296</td>
<td>1.334</td>
<td>0.203</td>
</tr>
<tr>
<td>Lymphocyte:RBC</td>
<td>0.209</td>
<td>1.131</td>
<td>0.277</td>
</tr>
<tr>
<td>T 30 min</td>
<td>-0.212</td>
<td>-1.089</td>
<td>0.295</td>
</tr>
<tr>
<td>PHA Swelling</td>
<td>-0.168</td>
<td>-0.868</td>
<td>0.400</td>
</tr>
<tr>
<td>SMR (W)</td>
<td>0.149</td>
<td>0.765</td>
<td>0.457</td>
</tr>
<tr>
<td>HVC NeuN+ cells</td>
<td>-0.148</td>
<td>-0.672</td>
<td>0.513</td>
</tr>
<tr>
<td>Immune PC1</td>
<td>0.121</td>
<td>0.649</td>
<td>0.527</td>
</tr>
<tr>
<td>CORT Dex</td>
<td>-0.139</td>
<td>-0.609</td>
<td>0.552</td>
</tr>
<tr>
<td>Body Size PCA</td>
<td>0.108</td>
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<td>0.598</td>
</tr>
<tr>
<td>Heterophil:RBC</td>
<td>0.075</td>
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<td>0.716</td>
</tr>
<tr>
<td>Immune PC3</td>
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<td>-0.364</td>
<td>0.721</td>
</tr>
<tr>
<td>CORT ACTH</td>
<td>-0.063</td>
<td>-0.332</td>
<td>0.745</td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td>0.052</td>
<td>0.270</td>
<td>0.791</td>
</tr>
<tr>
<td>H:L</td>
<td>0.052</td>
<td>0.261</td>
<td>0.798</td>
</tr>
<tr>
<td>T 60 min</td>
<td>-0.038</td>
<td>-0.198</td>
<td>0.846</td>
</tr>
<tr>
<td>PMR (W)</td>
<td>0.027</td>
<td>0.143</td>
<td>0.889</td>
</tr>
<tr>
<td>Metabolic Scope</td>
<td>-0.023</td>
<td>-0.120</td>
<td>0.906</td>
</tr>
<tr>
<td>Area X volume</td>
<td>0.022</td>
<td>0.114</td>
<td>0.911</td>
</tr>
<tr>
<td>RA Volume</td>
<td>-0.006</td>
<td>-0.032</td>
<td>0.975</td>
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</table>

Note: values in bold were significantly related to song stereotypy.
Table A-4 Results from step-wise multiple regression analysis showing the relationship between trill deviation and measures of adult phenotype in male song sparrows

<table>
<thead>
<tr>
<th>Measure</th>
<th>Beta</th>
<th>t statistic</th>
<th>p value</th>
</tr>
</thead>
<tbody>
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<td>PHA Swelling</td>
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<td>-2.910</td>
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</tr>
<tr>
<td>T 60 min</td>
<td>.325</td>
<td>1.663</td>
<td>0.119</td>
</tr>
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<td>PMR (W)</td>
<td>.331</td>
<td>1.621</td>
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</tr>
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<td>HVC volume</td>
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<td>-1.565</td>
<td>0.140</td>
</tr>
<tr>
<td>Metabolic Scope</td>
<td>.322</td>
<td>1.557</td>
<td>0.142</td>
</tr>
<tr>
<td>Immune PC1</td>
<td>-.328</td>
<td>-1.451</td>
<td>0.169</td>
</tr>
<tr>
<td>CORT ACTH</td>
<td>-.286</td>
<td>-1.375</td>
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</tr>
<tr>
<td>Heterophil:RBC</td>
<td>.235</td>
<td>1.151</td>
<td>0.269</td>
</tr>
<tr>
<td>RA Volume</td>
<td>.196</td>
<td>0.944</td>
<td>0.361</td>
</tr>
<tr>
<td>T 3 min</td>
<td>-.192</td>
<td>-0.888</td>
<td>0.390</td>
</tr>
<tr>
<td>H:L</td>
<td>.181</td>
<td>0.865</td>
<td>0.401</td>
</tr>
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<td>CORT restraint</td>
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<td>HVC NeuN+ cells</td>
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<td>Lean mass (g)</td>
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<td>0.595</td>
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<td>Body Size PCA</td>
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<td>Immune PC3</td>
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<td>0.485</td>
<td>0.635</td>
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<td>T 30 min</td>
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<td>0.474</td>
<td>0.643</td>
</tr>
<tr>
<td>CORT Dex</td>
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<td>0.459</td>
<td>0.653</td>
</tr>
<tr>
<td>Immune PC2</td>
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<td>0.329</td>
<td>0.747</td>
</tr>
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<td>Mass (g)</td>
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<tr>
<td>SMR (W)</td>
<td>.001</td>
<td>0.003</td>
<td>0.998</td>
</tr>
</tbody>
</table>

Note: value in bold was significantly related to trill deviation.

Discussion

I determined the relationship between adult song production and several measures of adult phenotype in individuals raised under control conditions or exposed to early-life stress. Although several previous studies have determined the effects of early-life stress on adult song production and other physiological and behavioural traits, this is the first study to examine the effects of early-life stress on adult song production and a host of
physiological traits in the same individuals and to determine if song predicts expression of these traits in adulthood. The measures of song production were significantly related to some of the measures of adult phenotype, most notably the swelling response to PHA, the song-control system, and in the case of syllable repertoire size regulation of the HPA and HPG axes. Many traits were not related to the measures of song production, particularly body size, body composition and metabolic rates. However, my results should be interpreted with caution since I included many measures of adult phenotype in my analyses and thus made many statistical comparisons, which could increase the likelihood of a type I statistical error. In addition, I also pooled birds from three different treatment groups, thus the correlations likely result from a combination of both within- and between-group effects. However, now that these relationships have been identified future studies can be conducted to look for more specific relationships.

Correlations between functionally independent traits in adulthood

There are multiple causal mechanisms that could explain correlations between two functionally independent traits in adulthood. First, the two traits may be developmentally correlated traits. This may occur if both traits develop in the post-hatch/natal period and if both traits are affected by variation in early environmental conditions (Spencer and MacDougall-Shackleton, 2011). Examples in the current study would include the positive correlations between the swelling response to PHA and song complexity, as well as the positive correlation between the CORT response to ACTH and immune PC1 scores ($r=0.482$, $p=0.023$, Table A-5 [at end of Appendix]), both of which were increased by early-life stress. This mechanism may be particularly important for static traits that are more or less fixed early in life. Second, two traits could be correlated
in adulthood due to variation in current conditions, as opposed to variation in developmental conditions. Thus two traits may be correlated in adulthood even if they are not affected by variation in the early environment. This mechanism may be particularly important for dynamic traits that show a large amount of plasticity in adulthood. Third, two traits could be correlated in adulthood because of genetic factors. That is, the expression of two traits may depend on common genes and an individual with “good genes” may be both a better singer and be of higher phenotypic quality. This would be another mechanism to explain how two traits that were not affected by early-life stress come to be correlated in adulthood, such as song stereotypy and immune PC2 scores in the current study.

Relationships between song and measures of phenotypic quality

The song-control system

I included measures of the song-control system in my analyses to provide an indicator of the quality of brain development and because several previous studies have found correlations between song production and volume of the song-control nuclei. However, it is unclear how exposure to early-life stress may affect these correlations. I found significant relationships between song production and the volumes of RA and HVC, but not Area X. First, I found that syllable repertoire size was positively related to the volume of RA. Consistent with this, past studies have found that the volume of RA is positively correlated with song complexity within species (Garamszegi and Eens, 2004). However, I did not detect the same relationship for song-type repertoire size. In Chapter 5, I found that both the volume of RA and syllable repertoire size were reduced after
exposure to early-life stress in song sparrows. In zebra finches, RA shows substantial
growth and development in the nestling and fledgling period when song acquisition
occurs (Bottjer et al., 1985; Bottjer et al., 1986). In addition, lesioning an area of the brain
that profoundly affects the morphology of the developing RA leads to abnormal song
development in zebra finches (Kittelberger and Mooney, 1999). These findings suggest
that RA may have an important role in song learning and raise the possibility that
impaired development of RA is responsible for the reduction in syllable repertoire size in
song sparrows. However, the results of the current study are correlational and future
studies are required to determine the causal relationship between early-life stress,
development of RA, and syllable repertoire size.

Second, I also detected a significant correlation between the volume of HVC and
both trill deviation and song stereotypy. Individuals with a larger HVC volume produced
higher performing trills (lower deviation from the performance limit) and also less
stereotyped song. To my knowledge, no previous studies have examined the relationship
between trill deviation and volume of the song-control nuclei. The finding that song
stereotypy was negatively correlated with HVC volume was surprising because previous
studies have found that the volume of HVC and song stereotypy are temporally correlated
in song sparrows, such that both peak in the spring (Smith et al., 1997). However, it was
not determined if the volume of HVC was significantly correlated to song stereotypy
within individuals in this study. Thus, although it is clear that expression of both traits
was greater in the spring it is not clear how these traits were related within individual
birds.

Body size, body composition, and metabolic rates
I did not detect significant relationships between the measures of song production and the measures of adult body size, body composition (lean or fat mass), or metabolic rates (SMR, PMR, or metabolic scope). In contrast, previous studies have found that song is significantly correlated to body size or condition. For example, in a study of free-living song sparrows, song complexity was positively correlated to body condition (Pfaff et al., 2007). In swamp sparrows, trill deviation is correlated to body mass, such that birds that produce higher performing trills (less deviation from the performance limit) are larger (Ballentine, 2009). To my knowledge, this is the first study to examine the relationship between song production and body composition or metabolic rates and my results suggest that song may not be an honest indicator of these traits in song sparrows. In Chapter 2, I found that early-life food restriction or CORT treatment did not affect any of these morphological and metabolic measures in male song sparrows (Schmidt et al., 2012b). In addition, nest identity (the natal nest of origin) was significantly related to body size, lean mass, fat mass, SMR, and PMR suggesting that these traits are strongly influenced by hereditary factors. In contrast, variation in many song attributes is largely dependent on the outcome of sensory learning processes and is heavily influenced by environmental factors, which may explain why song is not related to these traits.

The immune system

Song production was related to the swelling response to PHA and immune PC2 scores, but not immune PC1 scores, immune PC3 scores, or leukocyte profiles. Thus, song may be an honest indicator of some components of immune function in song sparrows. First, individuals with a larger swelling response to PHA, and thus a stronger immune response, had more syllables and song-types in their repertoires and also
produced higher performing trills. Indeed, the swelling response to PHA was the measure of adult phenotype that was most consistently related to adult song production (3 out of 5 song measures). Similarly, a previous study in free-living song sparrows found that the swelling response to PHA was positively correlated to song repertoire size (Reid et al., 2005a). The swelling response to PHA is considered a measure of the innate and the cell-mediated branch of the acquired immune system. The acquired immune system undergoes considerable development in the nestling and fledgling period as young birds encounter novel antigens in their environment (Palacios et al., 2009; Stambaugh et al., 2011). Thus, maturation of the acquired immune system shows substantial overlap with the period of song acquisition. In addition, I found that both the swelling response to PHA and song complexity were affected by exposure to early-life stress in song sparrows. Thus the swelling response to PHA and song complexity might be developmentally correlated traits.

Second, song stereotypy was negatively correlated to immune PC2 scores. Therefore, individuals with more stereotyped song had lower lysis titers and were less able to eliminate one strain of *E. coli* (8739). Both lysis of rabbit erythrocytes and microbicidal activity against *E. coli* 8739 are largely dependent on the complement system (Matson et al., 2005; Millet et al., 2007). This suggests that individuals with more stereotyped song may have less complement proteins. The direction of this relationship is surprising because males with more stereotyped song are typically considered to have a higher vocal performance and therefore would be expected to be of higher phenotypic quality and to have stronger immune systems. Thus the relationship between song and measures of constitutive innate immune function appears to be complex. Neither of these
traits were affected by early-life stress suggesting that this correlation is due to genetic factors or perhaps to variation in the current condition of adult males, as opposed to conditions during development. It is possible that males investing more in vocal performance have fewer resources to allocate to constitutive innate immunity (Lochmiller and Deerenberg, 2000). Future experimental studies are required to determine if there is a direct tradeoff between vocal performance and innate immunity in song sparrows.

**HPA axis**

The only significant relationship between HPA axis regulation and song was a significant correlation between the CORT response to DEX and syllable repertoire size. Individuals that were less able to suppress CORT in response to DEX, and who thus had less effective negative feedback, had more syllables in their repertoires. This finding is surprising because impairments in negative feedback of the HPA axis are associated with alterations in physiology and behaviour (Romero, 2004) and variation in negative feedback may predict fitness (Romero and Wikelski, 2010). Therefore, I would expect the highest quality males to have the most effective negative feedback and the most complex song repertoires, but I found the opposite relationship. However, the relationship between negative feedback and song complexity was no longer significant in the multiple regression analysis, suggesting that the significant correlation may have been driven by other variables.

The response of CORT to restraint or ACTH was not related to any measure of song production. In contrast, previous studies found that song complexity was negatively related to the response of CORT to restraint stress in free-living song sparrows.
(MacDougall-Shackleton et al., 2009; Schmidt et al., 2012a). In the present study, there was a trend for both syllable repertoire size ($r=-.400, p=0.072$) and song-type repertoire size ($-.406, p=0.068$) to be negatively correlated to the stress response. It is possible that this relationship would have been significant if the sample size had been larger, as it was in these previous studies. In addition, birds in my study exhibited very small increases in CORT in response to restraint compared to previous studies (MacDougall-Shackleton et al., 2009; Schmidt et al., 2012a). This is likely because subjects in my study were raised in captivity and habituated to handling. Perhaps with a different test stressor that induced a more effective increase in plasma CORT levels (e.g. exposure to a predator) I would detect a significant relationship between song complexity and the stress response.

**HPG axis**

Testosterone levels 30 min after injection of GnRH were significantly related to syllable repertoire size. Specifically, individuals with smaller increases in testosterone in response to GnRH had more complex syllable repertoires. Initial (unchallenged) testosterone levels and testosterone levels 60 min after injection of GnRH were not significantly related to the measures of song production. In song sparrows, regulation of testosterone via the HPG axis is an important determinant of aggressive behaviour. In support of this, simulated territorial intrusions (STI) rapidly increase testosterone levels during the breeding season when birds are actively establishing territory boundaries (Wingfield and Wada, 1989). Furthermore, male song sparrows implanted with testosterone behave more aggressively in response to an STI than do males that received control implants (Wingfield, 1984). My results suggest that syllable repertoire size may be an honest indicator of maximum testosterone levels, which may be achieved in
response to social challenges (Wingfield and Wada, 1989; Wingfield, 1985). A male with higher maximum testosterone levels may behave more aggressively, which could come at the cost of other activities such as parental care. Indeed, field studies have shown that testosterone treatment reduces parental care (Hegner and Wingfield, 1987; Ketterson et al., 1992), which would be important for females because biparental care is essential for the survival of offspring in many species of songbirds.

Conclusions

Consistent with previous studies, I found that song may be an honest indicator of multiple measures of adult phenotype in male song sparrows, including immune function and regulation of the HPA and HPG axes. However, many measures of adult phenotype were not related to song production, particularly measures of body size, body composition, and metabolic rates, which appear to be primarily influenced by genetic factors in my population of song sparrows. Combined with my previous studies, these results suggest that early-life stress can have long-term effects on song production and multiple physiological traits and that these traits may become correlated in adulthood as a result of the organizational effects of early-life stress. Thus song appears to be an honest indicator of a male’s early ontogeny in song sparrows and at least some features of their subsequent adult phenotype, but most certainly not all.
Table A- 5 Correlations between male song and physiological and behavioural traits

<table>
<thead>
<tr>
<th></th>
<th>Syllable Number</th>
<th>Song Type Number</th>
<th>Stereotypy</th>
<th>Learning Accuracy</th>
<th>Trill Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syllable Number</td>
<td>r=</td>
<td>p=</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.898</td>
<td>-0.011</td>
<td>0.455</td>
<td>-0.151</td>
</tr>
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<td>p=</td>
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<td></td>
</tr>
<tr>
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<td>p=</td>
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<td>Song type number</td>
<td>Stereotypy</td>
<td>Learning accuracy</td>
<td>Trill Deviation</td>
</tr>
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<td>-----------------</td>
<td>------------------</td>
<td>-------------</td>
<td>-------------------</td>
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<td><strong>-0.549</strong></td>
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<td>.193</td>
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<td>RA volume (mm³)</td>
<td>Area X volume (mm³)</td>
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<td>Mass (g)</td>
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<td>Body Size PCA</td>
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References


Curriculum Vitae

Education

Ph.D. Candidate (Biology) University of Western Ontario, London, ON 2009-present
Research Thesis: Song as an Honest Indicator of Developmental Stress in Song Sparrows
(supervisor: Dr. Scott A MacDougall-Shackleton)

M.Sc. (Neuroscience) University of British Columbia, Vancouver, BC 2007-2009
Research Thesis: Cortisol and Corticosterone in the Immune System and Brain of Developing Songbirds (supervisor: Dr. Kiran K Soma)

Research Thesis: Local Synthesis of Glucocorticoids in Neural and Immune Tissue of Developing Wild European starlings (supervisor: Dr. Kiran K Soma)

Scholarships and awards

• 2013 J.D. Detwiler Award
  $1500
• 2013 Michael Locke Graduate Travel Scholarship
  $500
• 2012 Journal of Experimental Biology
  $2,100, Travel fellowship
• 2012-2013 Queen Elizabeth II Graduate Scholarship in Science and Technology
  $15,000
• 2012 UWO Biology Department
  $500, Travel Award
• 2012 Robert and Ruth Lumsden Fellowship in Science
  $1,000
• 2011-2012 Queen Elizabeth II Graduate Scholarship in Science and Technology
  $10,000
• 2010 60th Meeting of Nobel Laureates in Lindau, Germany
  $2,000 from NSERC and Microsoft to attend meeting as a young researcher
• 2009-2011 Natural Science and Engineering Research Council of Canada
  $70,000, Canada Graduate Scholarship-Doctoral level
• 2009 UBC Graduate Program in Neuroscience
  $500, Travel Award
• 2008 UBC Faculty of Graduate Studies
  $400, Travel Award
• 2008 NSERC funded E-BIRD network
  $1,000, Travel Award for international collaboration
• 2007-2009 Natural Science and Engineering Research Council of Canada
  $35,000, Canada Graduate Scholarship-Master’s Level
Peer-reviewed journal articles


**Conference presentations**


Work experience

Teaching assistant

*University of British Columbia* 2007-2009
- Psychology 306, Animal Behaviour
- Psychology 314, Health Psychology
- Psychology 218, Research Methods and Introductory Statistics

*University of Western Ontario* 2010-present
- Biology 2290, Scientific Methods in Biology
- Biology 3440, Ecology of Populations
- Biology 3442, Conservation Biology

Field Assistant 2006

*University of British Columbia*

Research Assistant 2009

*University of British Columbia*