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Developmental Stress and the Effects on Physiological and Cognitive-Behavioural Traits in European Starlings

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Abstract

Birdsong is a complex, learned vocalization that is a phenotypic expression of male quality. The developmental stress hypothesis describes how the cost to possessing a high quality song is paid in early development. Stressful early-life experiences have adverse effects on the development of the neural circuitry that regulates song learning and production, which results in a male advertising with a low quality song in adulthood. The purpose of this thesis was to test the developmental stress hypothesis in several respects in European starlings (Sturnus vulgaris). My objectives were to assess the long-term effects of developmental stress on (1) physiological and cognitive-behavioural traits indicative of phenotypic quality in adulthood (Chapters 2, 3), (2) female songbirds’ auditory abilities and response to song (Chapter 4), and (3) male songbirds’ song behaviour and brain development across the lifespan in a species capable of learning new songs throughout adulthood (i.e., open-ended learner; Chapter 5). Juvenile starlings were reared in one of two developmental treatment conditions: control, or unpredictable access to food. The developmental treatment induced similar physiological effects in both sexes. Relative to controls, treatment birds had less fat during the developmental treatment, but following the treatment gained a significant amount of lean mass. In adulthood, aspects of endocrine regulation were also affected. Conversely, the effects on cognitive function differed between the sexes. Females reared in the treatment group were slower to acquire an auditory learning discrimination, while this effect was not observed in males. Treatment females were also less inclined to listen to conspecific (versus heterospecific) song in a behavioural choice paradigm. Furthermore, this behavioural response was mirrored by less neural activation in auditory forebrain regions when listening to conspecific song. Lastly, the developmental treatment did adversely affect
male song production and the supporting neural structures, but the effects diminished with age. Songbirds that are open-ended learners, compared to closed-ended learners (i.e., do not learn new songs in adulthood), may have a pattern of neural plasticity that allows for greater recovery from the effects of developmental stress. These results support the hypothesis and highlight the importance of sex and species differences.

Keywords

auditory learning, birdsong, cognition, developmental stress hypothesis, nutritional stress, song-control system, song preferences, European starling
Co-Authorship Statement

A version of Chapter 1 was published: Farrell TM, Kriengwatana B, MacDougall-Shackleton SA, 2015. Developmental stress and correlated cognitive traits in songbirds. *Comparative Cognition & Behavior Reviews*, 10, 1-23. Both Buddhamas Kriengwatana and Scott MacDougall-Shackleton wrote sections of the manuscript, and edited the entire manuscript. The sections from this manuscript used here are sections where I was the lead author.

A version of Chapter 2 was published: Farrell TM, Morgan A, Sarquis-Adamson Y, MacDougall-Shackleton SA, 2015. Effects of early-developmental stress on growth rates, body composition and developmental plasticity of the HPG-axis. *General Comparative Endocrinology*, 222, 134-143. Amanda Morgan and Yanina Sarquis-Adamson collected data and edited the manuscript. Scott MacDougall-Shackleton contributed to the experimental design, edited the manuscript, and provided funding for this project. I designed the experiment, collected data, analyzed data, and wrote the manuscript.

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Chapter 5 will be submitted for publication. Scott MacDougall-Shackleton will be a co-author. He contributed to the experimental design, edited the manuscript, and provided funding for the project. I designed the experiment, prepared and analyzed samples, analyzed data, and wrote the manuscript.

Chapter 6 was written by myself and is not published. All analyses within were performed by myself.
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“Flying is learning to throw yourself at the ground and miss.”

Douglas Adams
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CI: confidence interval
CV: coefficient of variation
CMM: caudomedial mesopallium
CORT: corticosterone
DHT: dihydrotestosterone
DLM: dorsolateral nucleus of the anterior thalamus
DPI: dots per inch
DR: discrimination ratio
GnRH: gonadotropin-releasing-hormone
HPA: hypothalamic-pituitary-adrenal
HPG: hypothalamic-pituitary-gonadal
LED: light-emitting diode
LMAN: lateral magnocellular nucleus of the anterior nidopallium
LMV: lamina mesopallium ventralis
LaM: lamina mesopallius
MBP: myelin basic protein
NCM: caudomedial nidopallium
nXIIIts: tracheosyringeal portion of the hypoglossal nucleus
OLS: ordinary least squares
PBS: phosphate buffered saline
PBST: triton in phosphate buffered saline
PCR: polymerase chain reaction
QMR: quantitative magnetic resonance
RA: robust nucleus of the arcopallium
SPL: sound pressure level
T: testosterone
ZENK: zif-268, erg-1, NGFI-A, Krox-24
Chapter 1

1 Introduction and Literature Review

Early-life environments have profound influence on shaping the adult phenotype. Specifically, stressful rearing environments have long-term consequences on adult physiology, neural functioning, and cognitive-behavioural traits. While developmental stress is well studied within the biomedical field, recent research in songbirds highlights similar findings in domesticated and non-domesticated species, opening up the field to answer questions with an ecological, and evolutionary focus. Birdsong is an excellent neurobiological model to answer these questions because it’s (1) a learned behaviour with well documented developmental periods, (2) supported by a specific neural circuitry, and (3) occurs in naturalistic contexts around reproductive events. In this chapter I will review the processes (ontogenetic, mechanistic, and functional) that shape birdsong. Then, I will discuss birdsong within the framework of the developmental stress hypothesis. In this thesis, the main objective was to address how unpredictable early rearing environments can alter the development of aspects of physiology, the cognitive abilities that support the learning and perception of birdsong, a female’s behavioural and neural response to song, and the production of song and neural development throughout the lifespan in open-ended song learners, European starlings (Sturnus vulgaris).

1.1 Birdsong

Many animals produce communicative vocalizations, although rarely is this a learned behaviour. Songbirds, along with humans, elephants, cetaceans, bats, hummingbirds, and parrots, learn to produce vocalizations (Doupe and Kuhl 1999). The
first studies with songbirds found support for a general pattern of song learning that distinguishes between two developmental phases: sensory acquisition and sensorimotor learning (Kroodsma and Miller 1996). Sensory acquisition occurs during a critical developmental window when the neural tissues that support song learning are poised to receive input (i.e., tutor song) from the environment. This sensory experience then generates a cascading neural and behavioural response, which leads to sensorimotor learning. Birds begin to produce song based upon a template that was generated, or activated, by auditory input received in the sensory acquisition phase (Marler 1997). At first, song during the sensorimotor phase is highly variable and is often compared to babbling in human infants (Aronov et al. 2008). However, auditory feedback allows birds to assess motor performance and consolidate changes to song performance until the produced song approximates the auditory template generated earlier (Konishi 1965; Fee and Goldberg 2011). This general pattern of song learning is supported unequivocally by disruption to either phase of song learning through such means as (1) varying the exposure to tutor song across the sensory phase (Thorpe 1958; Baptista and Morton 1981), (2) isolating young birds from hearing tutor song during the sensory phase (Marler 1981), and (3) deafening birds to inhibit auditory feedback during the sensorimotor phase (Konishi 1965; Nottebohm 1968).

The above model of song learning is well documented, but there is tremendous variation among the song learning patterns of the more than 4000 existing species of songbirds (Brenowitz and Beecher 2005). Most of our knowledge about song learning is derived from studies conducted on zebra finches (Taeniopygia guttata). In zebra finches, the sensory acquisition and sensorimotor phases overlap substantially, birds sing one
song type, and their song crystallizes by 90 days of age and remains unchanged in adulthood (reviewed in Slater et al. 1988). In several respects, zebra finches fall to one side in several song learning dimensions (Brenowitz and Beecher 2005). In contrast, other species extend the sensorimotor period later into life, such as white-crowned sparrows (Zonotrichia leucophrys nuttalli), who learn their songs in the first few months of life, but only begin to produce song the following breeding season (Marler 1970). While zebra finches are closed-ended leaners and sing but one song in adulthood, European starlings (Sturnus vulgaris) are open-ended song learners capable of learning in adulthood and sing several highly complex song types (Chaiken et al. 1994; Eens 1997).

1.2 Neural substrate for birdsong

Birdsong is controlled by a series of interconnected brain nuclei and pathways known as the song-control system (Nottebohm et al. 1976; Figure 1-1). Various studies have differentiated two primary pathways: the anterior forebrain pathway and the motor pathway (Brenowitz et al. 1997; Margoliash 1997). The anterior forebrain pathway is necessary for song learning, while the motor pathway is responsible for song production. HVC (proper name) is the nexus that links both pathways (Margoliash 1997). HVC sends efferent projections to the robust nucleus of the arcopallium (RA), which then innervates the tracheosyringeal portion of the hypoglossal nucleus (nXIIIts), as well as respiratory-control centers, to control the bird’s vocal organ, the syrinx, during singing. Information flows top-down from HVC and is organized hierarchically: HVC encodes higher-order song structure patterns than RA, and its neurons fire hundreds of milliseconds earlier than those in RA prior to the onset of song (Yu and Margoliash 1996). All constituent parts of this pathway are critical for song production. Lesions to either HVC or RA in adult birds
Figure 1-1 A schematic of the song-control system. A schematic of the song-control system. The anterior forebrain pathway is outlined in black arrows and the motor production pathway is outlined in gray arrows. DLM, dorsolateral nucleus of the anterior thalamus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium; and nXIIts, tracheosyringeal portion of the hypoglossal nucleus. Figure modified from Birdbrain by L. Shyamal based on Nottebohm F, retrieved from (https://commons.wikimedia.org/wiki/File:Birdbrain.svg). Used under Creative Commons Attribution 2.5 Generic license (https://creativecommons.org/licenses/by/2.5/deed.en) disrupts song production (Nottebohm et al. 1976; Simpson and Vicario 1990). The anterior forebrain pathway starts with HVC connecting to area X, to the dorsolateral anterior thalamic nucleus (DLM), to the lateral magnocellular nucleus of the anterior neostriatum (LMAN), and concludes by projecting to RA. Lesioning LMAN and area X in juvenile male zebra finches interferes with song acquisition, but has little immediate effect on song maintenance and production in adult male zebra finches (Bottjer et al.
1984; Sohrabji et al. 1990). Auditory neurons within LMAN and area X contain neurons that are highly responsive to song-selective information, specifically the bird’s own song, which enables the necessary auditory feedback for normal song development (Doupe and Konishi 1991; Doupe 1997). Lastly, while not part of the song-system, auditory forebrain nuclei (caudomedial mesopallium, CMM; caudomedial nidopallium, NCM) project to song-control nuclei and are necessary for the recognition and processing of song (Vates et al. 1996; Bolhuis and Gahr 2006).

Size and connectivity within the song-control system corresponds to behavioural differences in singing behaviour. This was first observed in noting that sex differences in singing behaviour correspond to sex differences in the brain: the sex difference is stark in species where females do not sing compared to species where both sexes sing (MacDougall-Shackleton and Ball 1999). Comparative analyses have found that species with larger repertoires tend to have proportionally larger HVCs than species with smaller repertoires (DeVoogd et al. 1993). Moreover, individuals within a species that have larger nuclei HVC and RA also have larger song repertoires and/or longer song bouts (reviewed in Garamszegi and Eens 2004). Thus species-differences, sex-differences, and individual differences all support a correlation between the anatomy of the song-control system and singing behaviour.

### 1.3 Song as indicator trait

Birdsong exudes the characteristics of a sexually selected trait: the behaviour is predominantly male and females show a clear preference for individuals with high quality song (Darwin 1871; Andersson 1994). Several hypotheses (e.g., information sharing in response to kin selection) have been applied to explain the function of birdsong, and the
sexual selection hypothesis is most strongly supported (Nowicki and Searcy 2014). As with the variation in song production, there is variation in female song preferences. Across species, the song characteristics that have the largest effect on females are song output, complexity and geographical (i.e., dialect) variation (Nowicki et al. 2002; Nowicki and Searcy 2005).

In general, female starlings prefer males that sing often and sing longer song bouts. For example, male starlings who sing longer song bouts pair up sooner in the breeding season, have larger clutches, and fledge more young (Eens et al. 1991; Mountjoy and Lemon 1996). Moreover, females preferentially choose males who sing longer song bouts in wild and captive preference tests (Eens et al. 1991; Gentner and Hulse 2000). Song output is usually linked to song complexity, another performance characteristic. Male starlings that sing long song bouts have larger repertoire sizes (Eens et al. 1991; Eens 1997), and song sparrows (*Melospiza melodia*) that sing more frequently through the day have more song types in their repertoire (MacDougall-Shackleton et al. 2009). Females of various species display more courtship behaviour in response to males with larger song repertoires (Searcy and Marler 1981; Baker et al. 1986) and larger syllable repertoires (Catchpole et al. 1984; Catchpole et al. 1986).

Lastly, geographical variation can arise in a species resulting in local dialects. Females prefer to mate with males that sing variants of their local dialect, even if they are presented with an unfamiliar singer (Searcy et al. 1997; Searcy et al. 2002). Though not a strict barrier, there is reduced gene flow between the dialect regions, which suggests that females mate preferentially with local males (MacDougall-Shackleton and MacDougall-Shackleton 2001).
Males with high quality song outperform males with lower quality song on a breadth of physiological and behavioural outcomes. For example, male starlings that sing longer song bouts exhibited stronger immune responses (Duffy and Ball 2002) and performed better on a spatial memory task (Farrell et al. 2011). Male song sparrows with larger repertoires hold territories longer, are in better body condition, pair up earlier in the breeding season, rear more chicks to independence, and exhibit greater inhibitory control (Hiebert et al. 1989; Reid et al. 2005; Pfaff et al. 2007; Boogert et al. 2011a). And male song sparrows that sang more syllables from the local dialect had fewer blood-borne parasites and fewer signs of physiological stress (Stewart and MacDougall-Shackleton 2008).

These findings exemplify an idea that many researchers have suggested for several decades – song quality is an indicator trait of male quality. Females prefer to mate with males who possess high quality song as they may receive direct (e.g., access to better territories, more parental care) and/or indirect (e.g., genes that confer advantages) benefits that will increase her reproductive success (Searcy and Andersson 1986). Males and females have conflicting reproductive interests. Males could manipulate females by inflating the quality of their signal, but females should only heed signals of reliable information (Dawkins and Krebs 1978). In this situation, if there was a cost associated with producing, or maintaining, the signal then the signal would be honest and reliable (Zahavi 1975). Otherwise, the signaler could deceive the receiver by inflating the trait to seem of higher quality. Historically, the cost of song was thought to lie with song production. A male who sings long, loud song bouts would require more energy (through the act of singing and/or being able to devote less time to foraging), or make themselves
more apparent to predators (Gil and Gahr 2002). However, these explanations could not account for variation in measures like repertoire size and dialects. In their influential paper, Nowicki et al. (1998) shifted the focus to the developmental costs associated with song learning. Next, I review how this paper has incited nearly two decades of research and expanded our understanding of the evolution and function of birdsong.

1.4 Developmental Stress Hypothesis

The prevailing hypothesis to explain how song can be an honest signal of male quality is the developmental stress hypothesis (Nowicki et al. 1998; Nowicki et al. 2000). Song learning and growth of the song-control system occur over a protracted period of early life, and may be influenced by the quality of the early-life environment. Thus, a bird that sings a high quality song is a bird that suffered relatively little stress during early life or was able to cope well with such stress. Consequently, song may also provide predictive information about other traits that are sensitive to developmental stress (Nowicki et al. 1998). Nowicki and colleagues (2002) first tested the hypothesis by restricting food-intake in nestling swamp sparrows (Melospiza georgiana) by feeding birds 70% on average of what their control siblings ate. Food-restricted birds copied songs less accurately from a tutor and had smaller RA volumes than controls. Since this study, this hypothesis has received substantial empirical support, as developmental stressors ranging from dietary manipulations, glucocorticoid administration, brood size manipulation, and immunological challenges have all been found to affect adult song and associated neurological structures in a variety of species (Table 1-1; Spencer and MacDougall-Shackleton 2011; MacDougall-Shackleton and Spencer 2012; Buchanan et al. 2013). However, not all experimental manipulations have found an effect of
developmental stress on song learning. This is particularly evident in the multitude of zebra finch studies, where the effects of a variety of developmental stressors on song learning are inconsistent, with some studies reporting effects (de Kogel and Prijs 1996; Spencer et al. 2003; Zann and Cash 2008; Holveck et al. 2008; Tschirren et al. 2009; Brumm et al. 2009) and others no effect (Gil et al. 2006; Kriengwatana et al. 2014).

1.4.1 Mechanisms of developmental stress

The exact reasons for such discrepancies between studies, especially with zebra finches, remain unclear because the mechanisms by which developmental stress affects song has yet to be firmly understood. Currently, developmental stressors are hypothesized to largely exert their effects by acting on the hypothalamic-pituitary-adrenal (HPA) axis. When stressors are perceived, the HPA axis mediates the physiological stress response by secreting glucocorticoids hormones (corticosterone [CORT] is the primary avian glucocorticoid). Thus, the way by which any type of developmental stressor affects song could be through activation of the HPA axis and the subsequent effects of CORT. For example, food-restriction can alter baseline and stress-induced levels of CORT in birds (Kitaysky et al. 2001; Pravosudov and Kitaysky 2006) and elevated CORT due to
Table 1-1 Summary of results from key experimental studies assessing the developmental stress hypothesis. The (-) symbol next to a trait is indicative that the manipulation caused a reduction in that behaviour/trait.

<table>
<thead>
<tr>
<th>Species</th>
<th>Manipulation</th>
<th>Effects on Song</th>
<th>Effects on Brain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swamp sparrows</td>
<td>Food restriction</td>
<td>- Copying accuracy</td>
<td>- HVC, RA volume</td>
<td>(Nowicki et al. 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Telencephalon volume</td>
<td></td>
</tr>
<tr>
<td>Zebra finches</td>
<td>Food restriction</td>
<td>- Song complexity &lt;sup&gt;a&lt;/sup&gt;</td>
<td>- HVC volume &lt;sup&gt;b&lt;/sup&gt;</td>
<td>(Spencer et al. 2003) &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Exogenous CORT</td>
<td>- Motif duration &lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>(Buchanan et al. 2004) &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Brood size</td>
<td>- No effect</td>
<td>No effect</td>
<td>(Gil et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>Brood size</td>
<td>- Copying accuracy</td>
<td></td>
<td>(Holveck et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>Diet quality</td>
<td>- Motif sound consistency</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>- Song rate</td>
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<tr>
<td></td>
<td></td>
<td>- Song complexity</td>
<td></td>
<td>(Naguib et al. 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Copying accuracy</td>
<td>(Zann and Cash 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Song sound consistency</td>
<td>(Brumm et al. 2009)</td>
</tr>
<tr>
<td>Song sparrows</td>
<td>Food restriction</td>
<td>- Song repertoire size</td>
<td>- HVC volume &lt;sup&gt;c&lt;/sup&gt;</td>
<td>(Honarmand et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>Food predictability</td>
<td></td>
<td>- RA volume &lt;sup&gt;d&lt;/sup&gt;</td>
<td>(MacDonald et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>Exogenous CORT</td>
<td>- Syllable repertoire size</td>
<td></td>
<td>(Schmidt et al. 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Copying accuracy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>European starlings</td>
<td>Food predictability</td>
<td>- Song bout length</td>
<td></td>
<td>(Buchanan et al. 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Song rate</td>
<td>(Spencer et al. 2004)</td>
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<td></td>
<td></td>
<td>- Repertoire size</td>
<td></td>
<td>(Farrell et al. 2011)</td>
</tr>
<tr>
<td>Bengalese finches</td>
<td>Brood size</td>
<td>- Song complexity</td>
<td></td>
<td>(Soma et al. 2006)</td>
</tr>
<tr>
<td>Canaries</td>
<td>Avian malaria</td>
<td>- Repertoire size</td>
<td>- HVC volume</td>
<td>(Spencer et al. 2005)</td>
</tr>
</tbody>
</table>

<sup>a, b</sup> Studies were conducted on the same group of zebra finches. The trait listed is marked with the appropriate reference.
<sup>c</sup> Studies that conducted analyses on nestling/juvenile birds. All other studies were conducted in adult birds.
<sup>d</sup> This effect was only detected in the birds that underwent the food-predictability treatment.
food-restriction can in turn adversely affect brain development (Welberg and Seckl 2001). In support of this, some studies have found parallel effects of CORT and food-restriction on song behaviour and the song-control system (Spencer et al. 2003; Buchanan et al. 2004; Schmidt et al. 2013b).

Nevertheless, food-restriction can also cause specific song and neural deficits not always seen in birds treated only with CORT. For example, song sparrows that were either treated with CORT or food-restricted both experienced a reduction in song complexity, but only food-restricted males were less accurate copying tutor song and had smaller volumes of the song-related brain nucleus RA (Schmidt et al. 2013b). This indicates that the influence of developmental stress on song may not necessarily be mediated by CORT per se, but by changing resource availability or resource allocation strategies of young birds. That is, birds that are deprived of nutrients early in life may have fewer resources to allocate towards brain development (Welberg and Seckl 2001), or may prioritize the development of certain systems when faced with limited nutrients (Schew and Ricklefs 1998). Differences in resource allocation can be mitigated by a strategy known as catch-up growth, which is a period of accelerated growth after a period of food-restriction (Metcalf and Monaghan 2001). Catch-up growth may be beneficial in the short-term by improving chances of survival, but there may life-long physiological debts incurred to paying the costs of this compensation (Metcalf and Monaghan 2001). Songbirds that are food-restricted in early-life often show slower growth rates, but typically these birds catch up to their control counterparts in adulthood once the stressor has been removed (Spencer et al. 2003; Krause et al. 2009; Krause and Naguib 2011; Schmidt et al. 2012; Kriengwatana et al. 2013). Collectively, these studies suggest that
glucocorticoid regulation is an important, but not the sole, mechanism by which developmental stress can have long-lasting impacts on birdsong. In fact, birds developing in challenging environments may direct resources towards the development of critical systems by altering glucocorticoid regulation (Wingfield et al. 1998).

1.4.2 Females and the Developmental Stress Hypothesis

Sexually selected traits have co-evolved in communications networks where there are signalers and receivers. In the context of the developmental stress hypothesis, the focus has largely been on the signalers (i.e., the males who sing). Variation in early-life environments affects song and the song-control system of males in a variety of species (MacDougall-Shackleton and Spencer 2012). To date, no study has assessed the effects of developmental stress on a male’s ability to perceive and respond to songs during development or in adulthood (but for a manipulation of the early tutoring environment see Sturdy et al. 2001). However, there are a limited number of studies examining how developmental stress affects females’ responses to songs, the majority of which have used zebra finches.

Data collected so far support the notion that females prefer the songs of control males to their stressed counterparts, and that female preferences may be altered by developmental stress (Spencer et al. 2005b; Riebel et al. 2009). Female zebra finches prefer the songs of control males to previously stressed males, which suggests that developmental stress alters songs in a biologically relevant fashion (Spencer et al. 2005b). In Riebel et al. (2009), female zebra finches raised in large broods (therefore presumed to have experienced more developmental stress) demonstrated equally strong preference for their tutor song as females from smaller broods. But, when given two
songs from unfamiliar males singing a ‘short’ or ‘long’ song based on motif duration, females from small broods demonstrated stronger absolute preferences for one song type (i.e., there was no consistent directionality of the preference; Riebel et al. 2009). In similar studies, females were found to prefer males whose developmental background matched their own – in song preference tests and live interactive mate choice trials the females from small broods preferred males from small broods, and vice versa for large brood females (Holveck and Riebel 2010; Holveck et al. 2011). However, food restriction early in life did not affect female zebra finch preferences for song complexity when given song from unfamiliar singers, but did reduce overall activity during mate choice trials (Woodgate et al. 2010; Woodgate et al. 2011).

Overall, these studies indicate that developmental stress can have some influence over a female’s response towards song (i.e., less motor activity), but there is no compelling evidence to suggest that developmental stress alters a female’s preference towards song based on measures of song quality alone (i.e., complexity, motif duration). Be that as it may, studies that have found preference effects also have a potential confound – the similarity of the stimulus songs to songs a female would have been exposed to during the sensorimotor phases of vocal development are often not accounted for. Exposure to song in early-life is a determining force to shaping female zebra finches song preferences (Riebel 2000; Lauay et al. 2004). A female raised in a large brood may acquire a preference for songs that share similar features to the songs of her development (i.e., similar to the song of her stressed father and/or developmentally stressed siblings). Therefore, rather than solely assessing if stress affects preferences for songs that vary in complexity, an additional consideration may be to assess preference for songs that vary in
features that sound more or less similar to the songs heard during the sensorimotor learning phase.

Apart from zebra finches, the only other species to date that has been studied with respect to the effects of developmental stress on female song preferences are song sparrows. Females that experienced food-restriction or CORT treatment show reduced preferences for conspecific song (versus a heterospecific song) compared to control females (Schmidt et al. 2013a). In addition, these stressed females showed patterns of neural activity in auditory forebrain areas (as measured by immunoreactivity of the immediate-early gene Zenk) that were not different when they listened to either conspecific or heterospecific song, while control females show significantly more immunoreactivity when listening to conspecific than heterospecific song (Schmidt et al. 2013a). This study suggests that female preferences are condition-dependent (Cotton et al. 2006) and may in part be caused by differences in neural activation in auditory forebrain regions in response to song (Schmidt et al. 2013a). Unlike the zebra finch studies, all song sparrows in the Schmidt et al. (2013a) study had the same exposure to tutor songs (a combination of live and tape tutors) in development and all stimulus songs were from males whose repertoires were unfamiliar. Therefore, differences in early-tutoring environments are not likely the cause of the weaker preferences seen in song sparrows.

1.5 Developmentally Correlated Traits

The central tenet of the developmental stress hypothesis can be extended to the development of other systems in addition to song learning. If song is an honest indicator of early-life conditions, then song could also be an honest indicator of the quality of other
physiological and cognitive systems that develop in parallel with song (Spencer and MacDougall-Shackleton 2011; Buchanan et al. 2013). Early-life environments are fundamental to shaping an organism’s phenotype (Metcalf and Monaghan 2001), and therefore developmental stressors are likely affecting several systems simultaneously. For instance, the neural systems that regulate song learning and spatial memory (song-control system and hippocampus, respectively) are functionally independent in the developing zebra finch (Bailey et al. 2009). Yet, these systems have developmental schedules that likely overlap (Clayton 1996; Brainard and Doupe 2002) and therefore could both be simultaneously affected by stressful environmental factors (Figure 1-2). If both systems are shaped by the same environmental factors (such as stressful rearing environments), this could result in them being correlated in adulthood despite their functional independence (Nowicki et al. 1998).

Cognition, as defined throughout this thesis, refers to the processes that underlie the acquisition, processing, and storage of information, which an animal uses to interact with its environment (Shettleworth 2009). It seems logical to assume that song, a process requiring cognitive abilities of memorization and learning, would be indicative of other cognitive abilities (Catchpole 1996; Nowicki and Searcy 2011). Several studies have found that song quality correlates with cognitive-behavioural measures such as inhibitory control in song sparrows (Boogert et al. 2011a), problem solving in zebra finches (Boogert et al. 2008), and spatial learning in European starlings (Farrell et al. 2011). Though, for every positive observed correlation there have been an almost equal number of null, or even negative, correlations. In the aforementioned study with song sparrows (Boogert et al. 2011a), there was no significant relationship between repertoire size and
Figure 1-2 Developmental stress may induce correlations among traits in adulthood. Horizontal orange arrows indicate the developmental trajectories of neurocognitive systems. If a stressor affects the development of multiple neurocognitive traits early in development (indicated by the lightning bolt), this may result in positive correlations between the traits in the adult animal (time point indicated by vertical blue arrow). In this way, traits are functionally independent in adulthood (e.g., birdsong and spatial memory) may be correlated across individuals. Figure modified from Spencer and MacDougall-Shackleton (2011) with permission.

performance on a color association task or a reversal task. Templeton et al. (2014) tested flocks of zebra finches on the same problem-solving task as Boogert et al. (2008), but did not replicate Boogert et al.’s results showing a positive relationship between song complexity and problem solving performance in zebra finches tested in isolation. Specifically, Templeton et al.’s (2014) study did not find any relationship between song complexity of solvers and non-solvers, nor between song complexity and latency to solve the task amongst solvers. The researchers cite responsiveness to social isolation as the reason underlying the correlation previously reported by Boogert et al. (2008). A separate
study in song sparrows found a negative relationship between spatial memory and song repertoire size (Sewall et al. 2013), to which the authors suggest could be the result of a trade-off between the development of song and spatial cognitive systems.

With the exception of Farrell et al. (2011), the other studies outlined had no explicit knowledge of the developmental history of the birds being assessed. Developmental history (i.e., the amount of developmental stress experienced) may contribute to differences in experience, motivation, and other factors that contribute to performance on cognitive tests (Thornton and Lukas 2012). To make the claim that song quality is predictive of other cognitive abilities, the onus must be placed on experimenters to demonstrate that these correlations are due to condition-dependent effects on the systems in question (Buchanan et al. 2013), which is difficult to accomplish when the developmental history of the subjects is unknown. Studying birds within the framework of the developmental stress hypothesis is a powerful experimental design to assess the relationship between song and other cognitive abilities. Song could be predictive of other cognitive processes if the development of neural substrates mediating song and other cognitive abilities overlap in time and are equally affected by developmental stress. Null, or negative, correlations between song and other cognitive traits could also be explained if the neural substrates supporting a particular cognitive ability are developing at different times than the song-control system, or if the development of neural/physiological systems that maintain more essential functions than song are canalized, or buffered from developmental stressors. And lastly, the relationship between song and other cognitive function could be related if they are, at least partly, mediated by common learning processes. If general learning processes that support song learning are adversely affected
by developmental stress, then song can act as an indicator of learning abilities in general. Ergo, a general intelligence factor should be detectable in songbirds.

General intelligence is a construct that captures cognitive performance across a series of tasks and suggests that performance on one task may be reflective of performance on other tasks (Deary et al. 2010). To date, there is evidence for general intelligence abilities in several species (Boogert et al. 2011b; Thornton and Lukas 2012). Large scale cognitive testing in songbirds however is in its infancy. Recent studies on bowerbirds that construct elaborate bowers, and sing courtship song, suggest that there is evidence for a general intelligence factor (Keagy et al. 2011b; Keagy et al. 2011a; Isden et al. 2013). However, in male satin bowerbirds (*Ptilonorhynchus violaceus*) measures of song quality did not correlate with individual problem-solving abilities (Keagy et al. 2011a). To date, the evidence from bowerbirds does not support the hypothesis that song quality is related to a general intelligence factor extracted from performance across various cognitive tests. Female bowerbirds appear to be make mating decisions using both measures of song and bower building in these species, which suggests that these traits are likely advertising different information (Candolin 2003).

Currently, the data support the notion that any general intelligence factors extracted from these studies reflect cognitive processes more or less independent of those necessary for song learning and performance. One limitation of the current research is that the experiments to date have focused solely on male populations, as the objective has been to assess correlations of cognitive abilities with a sexually selected trait. Future research must also study these relationships in females to better understand how sexual
selection may have shaped the evolution of complex cognitive abilities by females selecting mates based upon traits indicative of cognitive abilities (Boogert et al. 2011b).

1.6 Thesis Objectives

The primary objective of this thesis was to further the understanding of the proximate processes that influence the development, and ultimately, the adaptive function of birdsong. I conducted experimental tests of the *developmental stress hypothesis* to address unresolved issues within the field. My primary objectives were to assess the long-term effects of developmental stress on (1) physiological and cognitive-behavioural traits indicative of phenotypic quality in adulthood, (2) female songbirds’ auditory abilities and response to song, and (3) song production and the brain across developmental milestones in an open-ended song learning species.

I conducted all my experiments with European starlings (*Sturnus vulgaris*). In brief, I reared European starlings in an unpredictable environment by randomizing access to food. I chose starlings as my model species for several reasons. (1) Male song quality is an indicator of male quality. Several physiological, behavioural, and cognitive measures correlated positively with song quality (Duffy and Ball 2002; Buchanan et al. 2003; Spencer et al. 2004; Farrell et al. 2011). (2) Female starlings are attracted to male song, strongly prefer males with longer song bouts, and the strength of their preference is quantifiable with behavioural and neural responses that reflect variation in male song quality (Mountjoy and Lemon 1991; Mountjoy and Lemon 1996; Gentner and Hulse 2000; Gentner et al. 2001). (3) Starlings are open-ended song learners, and one of the few species for which this has been empirically demonstrated (Adret-Hausberger et al. 1990; Chaiken et al. 1994; Mountjoy and Lemon 1995). Lastly, (4) the developmental stress
manipulation used here has known adverse effects on immune function, stress responsiveness, dominance, spatial learning, and song (Buchanan et al. 2003; Spencer et al. 2004; Farrell et al. 2011).

1.6.1 Effects of developmental stress on physiological and correlated cognitive traits

A key prediction of the developmental stress hypothesis is that systems developing during the exposure to developmental stressors will also be affected, despite being functionally independent in adulthood (Spencer and MacDougall-Shackleton 2011). In Chapter 2, I first validated that my developmental manipulation was potent enough to induce a physiological response by measuring body mass and body composition of lean and fat tissue (1) during the developmental treatment, and (2) several weeks after the treatment ended to assess if birds utilized a compensatory growth strategy. I then subsequently studied how a developmental stressor known to affect song quality can affect other physiological and cognitive traits in adulthood. In Chapter 2, I also assessed the long-term effects of developmental stress on endocrine regulation of the hypothalamic-pituitary-gonadal axis (HPG), which is integral to regulating gonadal steroid hormones necessary for the regulation of breeding behaviours (Jawor et al. 2006; Jawor et al. 2007). In Chapter 3, I assessed the long-term effects of developmental stress in both males and females on two auditory learning tasks, a colour association task, and an inhibitory association task. I also assessed if performance on these learning tasks was correlated in order to determine if developmental stress affects cognitive abilities equally. Moreover, I assessed if auditory learning, a process associated with song learning and song perception (Woolley and Moore 2011), was correlated with other cognitive abilities.
Female songbirds are typically not considered in studies assessing correlated cognitive traits because song quality, a male-typical behaviour, is often the primary trait to which all other cognitive abilities are compared. Assessing auditory discrimination, instead of song quality, in conjunction with other cognitive tests (e.g., inhibitory control, association learning) allows the inclusion of females in the evaluation of whether developmental stress affects various cognitive processes equally.

1.6.2 Effects of developmental stress on females’ auditory responses and preferences

Possessing a song preference can be costly for female songbirds. A female that selects a male with a high quality song must develop the sensory organs and perceptual abilities that enabled her to evaluate the variation in quality across potential mates. There is strong experimental evidence in insects and fish that female preferences are dependent upon the condition of the female (Cotton et al. 2006). Weakened song preferences in female songbirds have been used as an indirect means to assess neural functioning. Yet, female preferences are influenced by a myriad of factors, several of which are external to a female’s condition (Cotton et al. 2006). I assessed the effects of developmental stress on female auditory abilities, song preferences, and neural functioning. In chapter 3, I evaluated auditory learning, independently of song preferences, in an operant conditioning paradigm testing both females and males. Then in chapter 4, I first evaluated females’ song preferences in an operant choice task. Subsequently, I assessed the volumes of song-control nuclei HVC, RA and area X, and induction of the immediate-early gene Zenk (an acronym for zif268, erg-I, NGFI-A, Krox24) in auditory forebrain regions (CMM, NCM) in response to male song.
1.6.3 Effects of developmental stress on behavioural and neural plasticity in an open-ended song learning species

Some species, such as European starlings, are capable of learning new songs into adulthood, but little is known about the mechanisms that allow for this to occur (Beecher and Brenowitz 2005). Studying the effects of developmental stress within an open-ended species across different developmental milestones may offer insight into the evolution of neural mechanisms in open-ended learners (Nowicki et al. 1998; Beecher and Brenowitz 2005; MacDougall-Shackleton and Spencer 2012). To date, the effects of developmental stress on song in open-ended song learners have only been quantified until the first breeding season (Buchanan et al. 2003; Spencer et al. 2005a; Farrell et al. 2011). It remains to be seen whether the adverse effects of early-life stress in an open-ended learner are potent enough to maintain a song quality deficit into subsequent breeding seasons. In chapter 5, I first assessed song quality of males during their first and second breeding season to see if early-life stress had pervasive deficits on singing throughout the lifespan or if song is more representative of recent conditions. Subsequently, I quantified the volume of song-control nuclei HVC, RA, and area X, and myelination of tracts within the song-control system at three points in development: 4 months of age, 1-year old, and 2-years old. This is the first experiment to document the effects of developmental stress on song-control system development in this species.
1.7 References


Chapter 2

2 Effects of early-developmental stress on growth rates, body composition and developmental plasticity of the HPG-axis

2.1 Introduction

Quality of the early-life environment is instrumental in shaping the developing phenotype, particularly for altricial songbirds that are born in an immature state. When environmental conditions are poor, physiological development may deviate from the normal trajectory, a process which is typically known as developmental plasticity (Debat and David 2001; McMillen and Robinson 2005). An evolved strategy to mitigate poor rearing conditions is to compensate once conditions improve by extending the developmental period through adjusting growth rates and resource allocation (Schew and Ricklefs 1998). For example, nestlings reared in food-restricted environments exhibit marked developmental plasticity in body mass growth. Growth is slow while the restriction is enforced, accelerated once it is removed (i.e., exhibit compensatory, catch-up, or accelerated growth), but ultimately these birds achieve adult weight comparable to nestlings reared under control conditions (Nowicki et al. 2002; Zann and Cash 2008; Brumm et al. 2009; Schmidt et al. 2012; Killpack et al. 2014; but see Pravosudov et al. 2005). Accelerating growth can be beneficial in the short-term, but there are costs associated with this strategy. For instance, in zebra finches (Taeniopygia guttata) accelerated growth has been linked in adulthood to reduced cognitive ability and altered exploration of new environments (Fisher et al. 2006; Krause and Naguib 2011), increased
resting metabolic rates (Criscuolo et al. 2008), and reduced resistance to oxidative damage (Alonso-Alvarez et al. 2007).

In the later juvenile period, physiological development continues after birds have achieved asymptotic size. Solely quantifying changes in overall body mass may fail to capture differential growth of body components (i.e., fat and lean mass) in the juvenile period. Through changes in feeding behaviour, diet quality and energy requirements, birds can undergo rapid physiological changes which may not be detected through total body mass (Dykstra and Karasov 1992; Piersma and Lindström 1997; Piersma et al. 1999; Lindström et al. 2000; Pierce and McWilliams 2004). Increases in lean mass results from growth of muscles, organs, feathers, and skeletal tissue (O’Connor 1977), while increases in fat mass suggest that surplus resources are being stored for later use (Reid et al. 2000; Ashton and Armstrong 2002). Recently, developmental studies have quantified changes in body composition to assess how birds allocate resources when food is restricted. These studies use destructive techniques (i.e., the bird is killed to complete the analysis) to illustrate which systems birds prioritize when faced with limited resources and/or are energetically more costly to develop (Killpack and Karasov 2012; Killpack et al. 2014). A limitation of destructive techniques is birds cannot be assessed repeatedly throughout development or into adulthood. Quantitative magnetic resonance (QMR) allows the accurate, rapid, and repeatable assessment of an animal’s body composition via nondestructive means (Guglielmo et al. 2011; McWilliams and Whitman 2013). A recent study using QMR in zebra finches found that the largest determinant of body fat later in life was attributed to nutritional conditions during the juvenile period, more so than nutritional conditions of the nestling period (Kriengwatana et al. 2013). QMR is an
excellent tool to quantify long-term differences in growth patterns when birds are faced with developing in challenging environmental conditions.

In mammals, and to a lesser extent birds, environmental stressors exert organizational effects on the hypothalamic-pituitary-adrenal (HPA) axis and glucocorticoid production (McMillen and Robinson 2005; Lupien et al. 2009; Schoech et al. 2011). Glucocorticoid regulation is intimately linked with the regulation of another important endocrine system: the hypothalamic-pituitary-gonadal (HPG) axis (Rivier and Rivest 1991; Viau 2002). In mammals, stressors can alter HPG axis function, resulting in reduced gonadal steroids and, thereby, altered reproductive function (Tsigos et al. 1999; Nepomnaschy et al. 2004; Hardy et al. 2005; Kyrou and Tsigos 2008). The avian HPG axis largely develops in the embryo and the first few weeks of life, but exhibits plasticity several months after hatch and across reproductive years (Ottinger and Bakst 1995; Sockman et al. 2004). Androgens, such as testosterone (T) and dihydrotestosterone (DHT), are gonadal steroid hormones that modulate physiological and behavioural traits necessary for reproduction (Balthazart 1983; Wingfield et al. 1990). For example, increased T levels are associated with better song quality and enhanced immune function in male European starlings (Sturnus vulgaris; Duffy and Ball 2002; Ball and Balthazart 2010) and an increased likelihood of acquiring and maintaining a breeding site in female spotless starlings (Sturnus unicolor; Veiga and Polo 2008). However, elevated T levels are also associated with reduced parental care (Eens et al. 2007). In birds, only one study in song sparrows (Melospiza melodia) has examined how early developmental experiences may program HPG axis function into adulthood: males treated with glucocorticoids early in life had higher baseline T levels, while females treated with
glucocorticoids, or subjected to food-restriction, had lower estradiol levels than control females (Schmidt et al. 2014). Thus, a stressful rearing environment may alter a bird’s allocation of resources, such that reproductive function is altered through changes to HPG axis function.

In the current study, I reared juvenile starlings on an unpredictable food supply treatment that is known to have detrimental effects on several physiological, neural and behavioural measures in starlings (Buchanan et al. 2003; Farrell et al. 2011) and song sparrows (Schmidt et al. 2012; Schmidt et al. 2013; Schmidt et al. 2014). I monitored the effects of the treatment on body mass and body condition during, and following, the treatment period to determine if unpredictable food access altered growth of fat and lean tissue. In adulthood, I assessed the long-term effects on body mass and quantified the size of testes in adult males while they were in breeding condition. For both sexes, HPG axis function was measured by assessing androgen levels using a gonadotropin-releasing hormone (GnRH) challenge. A GnRH challenge allows for the assessment of reproductive condition of the animal (Wingfield et al. 1979), as well as inference of the hormonal response an individual would issue in a socially challenging situation (Jawor et al. 2007). I predicted birds raised in the treatment group would grow more slowly during the treatment, faster once the treatment ceased (i.e., exhibit accelerated growth), but there would be no long-term effect on adult body mass. I also predicted that birds from the treatment group would have less fat and lean mass during treatment, but would compensate after the restriction by gaining more fat and lean mass. I predicted that male and female birds from the treatment group would have reduced androgen production compared to controls. Furthermore, as accelerated growth is known to be associated with
costs later in life (Metcalfe and Monaghan 2001), I predicted that individuals that accelerated growth the fastest would have the lowest androgen production in adulthood.

2.2 Methods

2.2.1 Subjects and treatment

I collected starlings as nestlings around London, Ontario (42°98’ N, 81°25’ W) during May and June 2012. A total of 55 nestlings from 15 nests (range: 1-6 birds per nest) were brought into captivity at an average of (± SD) of 13.0 ± 3.9 days. Shortly after capture, I took a small blood sample (< 50 µL) by piercing the brachial vein with a 26-gauge needle, extracted the DNA from whole blood, and used polymerase chain reaction (PCR) to genetically sex all birds (Griffiths et al. 1998). I housed all nestlings by nest in small bowls lined with tissue paper placed inside a cage (76 X 46 X 45 cm). I fed nestlings a hand-rearing diet, a mixture of commercial chick starter, wheat germ, carrots, hardboiled egg, grit, and vitamins (Prime avian vitamin supplement, Rolf C. Hagen Inc, Montreal, QC, Canada), ad libitum until the birds were feeding independently. I then transitioned birds to chick starter feed and separated birds into individual cages (same size as above) at 33.2 ± 1.7 (SD) days of age. At this time, within each nest, I randomly assigned birds to control or unpredictable conditions (i.e., the treatment group) counterbalancing across sex (see Table 2-1).

I used an unpredictable food supply treatment from 35-115 days of age to induce a period of food restriction. In brief, control birds were given ad libitum food (chick starter), while birds in the treatment group had their food removed for a set interval, the time of which would occur randomly between 09:00-17:00 h. Female starlings are smaller than males (Feare 1984), therefore I scaled the interval of food-restriction to be
Table 2-1 Samples size by treatment condition and sex across the different phases of the experiment

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Sex</th>
<th>Treatment Period</th>
<th>Post-treatment Period</th>
<th>GnRH Challenge + Testis Volume (Males only)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Male</td>
<td>17</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>9</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Treatment</td>
<td>Male</td>
<td>17</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>55</td>
<td>39</td>
<td>37 (17)</td>
</tr>
</tbody>
</table>

Eight males from both control and treatment groups were sacrificed on the final measurement day of the treatment period for a separate study. One control female and one treatment male died before the GnRH challenge and testis volume measurements could be taken.

3 h and 4 h for females and males, respectively. When I removed the food cups, I ensured that loose food that had spilled in the cage was also removed. To control for disturbance, I also opened the cages of controls birds at the same time. Birds in both groups were supplemented with 5-10 mealworms daily. Once the treatment period was over, treatment birds were given *ad libitum* access to food and both groups were given mealworms 3 times a week. Throughout the developmental treatment, birds were maintained on a light:dark cycle consistent with the summer season photoperiod (LD 15.5:8.5).

Between the ages of approximately 6-12 months, a subset of birds (Table 2-1) in this study participated in a series of cognitive experiments that manipulated food intake (Chapter 3; Farrell et al. 2015a). In brief, auditory abilities were assessed in an operant-conditioning paradigm (see Sturdy et al. 2001). To ensure high motivation during testing, birds were food-restricted to 85-90% of their *ad libitum* body weight. While unintentional, this period of food-restriction was an additional stressor in the months prior to the first breeding season. I have included it as a factor for all statistical analyses for traits assessed in adulthood (i.e., adult body mass, male testis volume, and all
Table 2-2 Sample sizes of participation in the cognitive experiments (Chapter 3) presented by treatment condition and sex.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Sex</th>
<th>Participated in the cognitive experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Control</td>
<td>Male</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>5</td>
</tr>
<tr>
<td>Treatment</td>
<td>Male</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>22</td>
</tr>
</tbody>
</table>

measures from the GnRH challenge). Hereafter, I will use the terms “treatment” and “cognitive treatment” to delineate any effects caused by the developmental treatment or as a result of food manipulations experienced while participating in the cognitive treatments, respectively.

2.2.2 Body composition

I took a series of body measurements prior to, during, and after the treatment period. During the treatment period, measurements occurred every tenth day for a total of 8 measurement days. Measurements were taken between 1300 and 1500 h on these days. On measurement days, the food-restriction treatment was suspended for the day, birds were given a bath and their cage was cleaned. After the treatment period ended, birds underwent two follow-up measurement days at 10 and 35 days post-treatment when they were approximately 125 and 150 days old. Birds were also weighed as adults during the GnRH challenge.

2.2.2.1 Body condition measures

I weighed birds to the nearest 1g prior to starting the treatment, and on all subsequent measurement days during and following the treatment. Tarsus was measured on both legs to the nearest 0.1mm with dial calipers prior to the start of treatment. At this
age, tarsus length is considered fixed and has grown to full adult size (Feare 1984; Smith 1993). Average tarsus length was used in further statistical analysis as an indicator of skeletal size.

2.2.2.2 Body composition analysis

Using quantitative magnetic resonance (QMR) analysis (Guglielmo et al. 2011), I assessed fat and lean mass every measurement day and during the two post-treatment measurement days. The QMR is a custom-designed instrument for use with small birds (Echo-MRI-B, Echo Medical Systems, Houston, Texas). I validated the instrument daily using 5 g and 94 g canola oil standards. For each measurement I placed birds into a plastic holding tube and inserted them into the instrument for two consecutive scans (approximately 128 s total duration). I averaged the values of the fat and wet lean mass measurements from the two scans to the nearest 0.001g. Fat and lean mass measurements were adjusted using calibration equations obtained by ordinary least squares (OLS) regression for predicting gravimetric body composition using body composition data collected through QMR developed with European starlings \( \text{fat mass: } 0.62 + 0.95(\text{QMR raw value}); \text{ lean mass: } 9.06 + 1.07(\text{QMR raw lean value}) \}). A previous study validating the QMR showed that coefficients of variation for fat and lean mass were \( \pm 2.4 \) and \( \pm 0.7\% \), respectively; relative accuracies for fat and lean mass were \( \pm 10.1 \) and \( \pm 1.5\% \), respectively (Guglielmo et al. 2011).

The QMR instrument was unavailable until early June, and therefore I was only able to scan a subset of the birds prior to treatment beginning at 35 days of age \( (n = 10) \) and on the first measurement day at 45 days of age \( (n = 20) \). All birds were scanned on the remaining measurement days through 55 to 115 days of age \( (n = 55) \). At the end of
treatment, a subset of males from both the treatment and control groups were euthanized for a separate study (see Chapter 5), but the remaining birds (n = 39) were weighed and scanned for the two post-treatment measurements at 125 and 150 days of age (see Table 2-1).

2.2.3 GnRH challenge

2.2.3.1 Blood sampling and injections

I characterized the HPG-axis response to a GnRH challenge following protocols used in other species (Jawor et al. 2006; Jawor et al. 2007; Schmidt et al. 2014). This protocol allowed for baseline, peak and integrated testosterone levels to be measured. To determine the appropriate dose of GnRH, I conducted a pilot study using 5 adult starlings (3 males, 2 females) that were not part of this study. These birds had been held in captivity since 2008 and were photostimulated at the time of the pilot. Subjects received either a low (0.05 µg GnRH/g of body mass) or high (0.10 µg GnRH/g of body mass) dose of GnRH, and then a week later received the other dose. Order of dose was counterbalanced across subjects. Although there was no significant difference in androgen levels across the doses ($F_{1,30} = 0.03$, $P = 0.86$), I chose to use the high dose as values in the low dose condition were still rising at 60 minutes (Figure 2-1).
Figure 2-1 Plasma androgen levels measured in adult starlings during a pilot study to determine the appropriate dose of gonadotropin-releasing hormone (GnRH) to use for the GnRH challenge. After collecting baseline samples, birds were injected with either a low dose (0.05 µg/g i.m.) or high dose (0.10 µg/g i.m.) and plasma samples were collected 30 and 60 minutes post-injection. Points not sharing a letter are significantly different for \( P < 0.01 \).

To summarize the challenge procedure, birds were captured, the brachial vein was pierced with a 26-gauge needle, and a blood sample was collected within 3 minutes into heparinized micro-hematocrit tubes. I then injected birds with a 0.10 µg GnRH/g of body mass dose of chicken GnRH-I (54-8-23, The American Peptide Company, Inc., Sunnyvale, CA, U.S.A.) into the pectoralis muscle. Birds were then placed in an opaque cloth bag and two more blood samples were collected 30 and 60 minutes post-injection. I collected 100 µL of blood at each time point for a maximum 300 µL of blood taken from each bird. All blood samples were collected between 12:30 and 14:30 h. After sample collection, I immediately centrifuged samples at 13,000 g for 10 min, collected the plasma, and stored them at -30 °C until processing.

Males were tested at approximately 14 months of age [438.1 ± 13.4 (SD) days of age], while females were tested at approximately 18 months of age [546.0 ± 25.1 (SD) days of age]. Females were tested at an older age because of experiments assessing the
effects of developmental stress on song preferences (Chapter 4; Farrell et al. 2015b). Both males and females were held on a short day photoperiod (LD 10:14) for several months [Males: 90.7 ± 3.5 (SD) days; Females: 186.1 ± 13.7 (SD) days] in order to dissipate photorefractoriness (Dawson and Goldsmith 1983). Then, birds were photostimulated by being placed on a long day photoperiod for approximately 26.2 ± 1.7 (SD) days. Specifically, males were placed in outdoor aviaries and were subjected to the natural photoperiod for London, Ontario from the dates of June 17, 2013 until August 2, 2013 (photoperiod ranged from a high of ~LD 15.5:8.5 to a low of ~LD 14.5:9.5). During photostimulation, male subjects were housed with a few other males, except for the day prior to the GnRH challenge when each male was paired with one adult female not part of the study for a courtship song assessment (Chapter 5). On the day of the GnRH challenge, each male was housed individually, but had visual and auditory contact with other birds. Females were held indoors for the duration of the study, and were therefore on a controlled long day photoperiod of (LD 15:5:8.5) for 24.7 ± 1.7 (SD) days. During this time, their song preferences were being assayed for a separate study (Chapter 4; Farrell et al. 2015b). All birds were tested after several weeks of exposure to long days and I confirmed that females were in breeding condition by observing the presence of enlarged ovaries with a visible follicular hierarchy. For males, breeding condition was confirmed by measuring the volume of both testes (explained below). All birds were fed ad libitum for several months prior to and during the GnRH challenge.

2.2.3.2 Androgen assay

Androgen levels were quantified using a commercially available enzyme immunoassay (Expanded Range Salivary Testosterone Enzyme Immunoassay Kit 1-
This kit has been used previously to quantify androgens in plasma of a variety of songbird species (Washburn et al. 2007; Vandermeer 2013; Schmidt et al. 2014); however, it had not been previously used in starlings. To validate this kit for use with starlings, I serially diluted a pooled starling plasma sample and compared the results to the standard curve using an ANCOVA (Chard 1995). The interaction term between the serial dilution and the standard curve was not significant ($F_{1,8} = 2.78, P = 0.13$; Figure 2-2), which indicates that the line slopes are not statistically different (Newman et al., 2008) and that the kit is effective for measuring androgens in starling plasma. I followed the manufacturer’s instructions exactly; with the exception that I diluted 15 µL of plasma into 60 µL of assay buffer, and analyzed each sample in

![Graph](image)

**Figure 2-2** Serially diluted samples of pooled European starling plasma in relation to the androgen standard curve. The lines are parallel and do not differ statistically, indicating that the enzyme immunoassay kit used to detect androgens is valid for use in European starlings.
duplicate by adding 25 µL of diluted plasma to each well. All samples were initially randomized by treatment and sex across three assays. However, 10 samples registered outside the upper limit of the standard curve. I reanalyzed these samples in a fourth assay by further diluting 10 µL of plasma into 190 µL of assay buffer, and each sample was analyzed in duplicate by adding 25 µL of the diluted plasma to each well. In each assay a low control, high control, and a pooled starling plasma sample were included. The inter-assay coefficient of variation (CV) was 11.65% for a low control, 1.25% for a high control, and 2.62% for a pooled plasma sample. Intra-assay CVs were 1.99%, 5.24%, 7.97% and 10.66%. Androgen levels for all samples fell within the range of the standard curve, except for 4 samples from female birds. These 4 values were assigned a value of the detection limit of the assay kit, which was defined as 2 standard deviations above a blank control (3.56 pg/mL).

### 2.2.4 Testis volume

Immediately after the GnRH challenge, all males were euthanized by isoflurane overdose and were subsequently decapitated. Brains were extracted for a separate study (Chapter 5). I immediately dissected out both testes and recorded their width and length to the nearest 0.1 mm with dial calipers. I calculated the volume of each testis using the equation for the volume of an ovoid sphere, $V = \frac{4}{3} \pi a^2 b$, where $V$ is the volume, $a$ is the radius, and $b$ is half of the length of the testis (Perfito et al. 2008). The average of the two testis volumes was used in all statistical analysis.
2.2.5 Data and statistical analysis

All data were analyzed using linear mixed models. I examined the effects of the developmental condition (i.e., treatment), sex, and their interaction (when appropriate) as fixed effects in all models. Models were subsequently tailored to each experiment by adding covariates and additional fixed effects of interest, along with any of their higher order interactions with treatment and sex. Subject and nest of origin were entered as random effects in all models. I only report the results of random effects when they remained in the final model.

For body mass, I conducted 3 separate models assessing body mass as the dependent variable: (1) during the treatment period, (2) the two measurements taken post-treatment, (3) and adult body mass measured during the GnRH challenge. Tarsus length on the first day of the developmental treatment was entered as a covariate in all models. Age during the treatment period was entered as a repeated effect (auto-regressive heterogeneous covariance structure) and a fixed effect. During the post-treatment period, age was entered as a repeated effect (compound symmetry covariance structure) and fixed effect. Adult body mass was measured once and therefore there were no repeated effects for that model; birds participation in cognitive experiments was included as a fixed effect for adult body mass.

For body composition, I ran separate models assessing fat mass and lean mass as the dependent variables: (1) during the treatment period, and (2) during the post-treatment measurements. Age during the treatment period was entered as a repeated effect (auto-regressive heterogeneous covariance structure) and a fixed effect. During the post-treatment period, age was entered as a repeated effect (compound symmetry
covariance structure) and a fixed effect. To control for the fact that structurally larger birds will have more mass, we included tarsus length as a covariate in all models. And, with regards to the models assessing fat mass, I also included lean mass as a covariate to control for the fact that birds that weigh more will also have more fat.

For the GnRH challenge, I conducted separate analyses for both males and females as each challenge was conducted at different ages and in different environmental conditions (i.e., males were housed socially outdoors, while females were held in individual cages indoors). I conducted 3 models on each of the following dependent measures: (1) baseline androgen levels (0 minute), (2) peak androgen levels (30 minute - baseline sample), and (3) integrated androgen levels (calculated as the area under the gonadal response curve). I included fixed effects of developmental treatment, participation in cognitive experiments, and their interaction. Adult body mass was entered as a covariate. In addition, I also included the growth rate (calculated as daily body mass gain; g/day) for the period of time when accelerated growth would have been most pronounced (from last day of treatment until 10 days post-treatment) as a covariate to assess the relationship between growth rate and HPG axis functioning. All data were log-transformed to meet assumptions of normality.

For testis volume, I analyzed the average testis volume for males as the dependent variable. In addition to developmental treatment, I also included if birds participated in the cognitive experiments. Adult body mass was also included as a covariate to control for overall body size.

I first constructed fully loaded models (all predictors and interactions included) and then calculated the minimal adequate model by removing non-significant ($\alpha > 0.05$)
predictors to maintain parsimony and improve model fit (West et al. 2007). Models were compared to each other by likelihood ratio tests, following a ‘smaller is better’ form. In brief, the difference in -2 restricted log-likelihood values and number of parameters between models was calculated. The difference in values is then assessed against a chi-square distribution (the difference in -2 REML log-likelihood values is the test statistic and the difference in parameters is the degrees of freedom). If by removing a parameter, the difference between models results in a test statistic larger than what the chi-square table would predict (based on the probability value $\alpha = 0.05$), than this signified that the parameter in question explained a significant portion of variation within the data and should be retained in the final model. If removing the parameter did not alter the fit above the probability value $\alpha = 0.05$ criteria, than the parameter was removed to obtain the most parsimonious solution.

All significant results reported here are the minimal adequate models with restricted maximum likelihood estimation and post-hoc tests were carried out using Bonferoni corrections. Statistics reported for non-significant effects are the values from the full model prior to being removed. I checked residuals for each final model against a normal distribution and found no violations of normality. SPSS software (v. 21) was used for all analyses.

2.3 Results

2.3.1 Body mass

2.3.1.1 During treatment

Body mass did not differ between developmental treatments ($F_{1,98.2} = 0.33, P = 0.57$; Figure 2-3a). Males weighed significantly more than females (Sex: $F_{1,105.5} = 19.67, P <$
0.0001; estimated marginal means - Males: 80.80 ± 0.68 g; Females: 77.84 ± 0.76 g), but the sex by treatment interaction was not significant \((F_{1,101.5} = 3.51, P = 0.06)\). There was a significant effect of age \((F_{8,187.7} = 47.09, P < 0.00001)\), but there was no significant a higher-order interaction between the variables sex, treatment, and age (all \(P_s > 0.27)\). Birds with a bigger tarsus weighed significantly more \((F_{1,104.5} = 26.09, P < 0.00001)\).

Nest of origin remained in the final model \((Wald Z = 1.88, P = 0.06)\).

### 2.3.1.2 Post-treatment

Following the end of the treatment period (125 and 150 days of age), treatment birds weighed more than controls \((F_{1,23.2} = 11.81, P < 0.01;\) Figure 2-3a). As during the treatment, males weighed more than females \((F_{1,30.8} = 8.54, P < 0.01)\) and birds with longer tarsus weighed more \((F_{1,32.5} = 32.05, P < 0.00001)\). There was no significant effect of age \((F_{1,37.00} = 0.03, P = .09)\), and none of the higher order interaction between sex, treatment and age were significant (all \(P_s > 0.10)\). Nest of origin remained in the final model \((Wald Z = 1.71, P = 0.09)\).

### 2.3.1.3 Adulthood

In adulthood, several months after the developmental treatment ended, there was no significant difference in overall body mass between the treatment groups \((F_{1,37} = 0.39, P = 0.54)\); Control: 76.49 ± 1.02 g; Males: 75.64 ± 0.95 g). There was a sex difference \((F_{1,33} = 7.01, P = 0.012)\), but unlike during the developmental treatment, adult females weighed more than adult males \((\text{Females}: 77.78 ± 0.89 g; \text{Males}: 74.30 ± 0.97 g)\). Participation in the cognitive experiments was also a significant effect \((F_{1,33} = 9.75, P < 0.01)\). Birds that participated were significantly heavier than birds that did not
Figure 2-3 Effects of developmental treatment (control versus treatment) on (a) body mass, (b) lean mass, and (c) fat mass during the treatment period and the two post-treatment measures taken at 125 and 150 days of age. All data points are means (error bars representing ± SE) as calculated including covariate tarsus length and random effect of nest of origin. Fat mass also includes covariate of lean body mass. All data points for the treatment group have been offset to the right to prevent overlap of data points. ** $P < 0.01$, *** $P < 0.001$. 
Participation: 78.12 ± 0.84 g; No participation: 73.95 ± 1.03 g). Tarsus was a significant covariate ($F_{1,33} = 7.92, P < 0.01$) with birds with longer tarsi weighing more. Participation in the cognitive experiments did not significantly interact with sex ($F_{1,37} = 3.76, P = 0.06$) or developmental treatment ($F_{1,37} = 3.52, P = 0.07$). All other higher-order interactions were not significant (all $P$s > 0.16).

### 2.3.2 Body composition

#### 2.3.2.1 During treatment

During treatment, there was no significant difference in lean mass between treatment groups ($F_{1,87.7} = 2.28, P = 0.14$; Figure 2-3b). However, there was a difference with regards to fat mass ($F_{1,79.4} = 12.28, P < 0.001$; Figure 2-3c). Control birds had significantly more fat throughout the developmental treatment period than treatment birds (Control: 5.27 ± 0.18 g; Treatment: 4.78 ± 0.17 g). Males had more lean mass than females (Sex: $F_{1,72.6} = 16.92, P < 0.001$; estimated marginal means - Males: 62.82 ± 0.63 g; Females: 65.31 ± 0.56 g), but the sexes did not differ in their amounts of fat mass ($F_{1,98.5} = 0.08, P = 0.77$). For both fat and lean mass, there was no significant interaction between sex and developmental treatment (Lean: $F_{1,72.06} = 0.57, P = 0.45$; Fat: $F_{1,82.8} = 3.25, P = 0.08$). Age was significant effect in both models for lean and fat mass (Lean: $F_{8,106.7} = 23.82, P < 0.0001$; Fat: $F_{8,64.8} = 16.65, P < 0.0001$) and there was a significant interaction between sex and age for lean mass ($F_{8,91.0} = 2.97, P < 0.01$). Birds with a bigger tarsus had significantly more lean mass ($F_{1,87.7} = 33.61, P < 0.0001$), but significantly less fat mass ($F_{1,112.4} = 13.97, P < 0.001$). In general, lean mass was positively related to the amount of fat mass a bird had ($F_{1,270.4} = 114.60, P < 0.0001$). No other higher-order interaction between the variables sex, treatment, and age were
significant for the lean or fat mass model (all $P$s > 0.18). Nest of origin remained in both final models (Lean: Wald $Z = 1.84$, $P = 0.07$; Fat: Wald $Z = 1.80$, $P = 0.07$).

### 2.3.2.2 Post-treatment

Following the treatment period, treatment birds had more lean mass than controls ($F_{1,21.6} = 19.39$, $P < 0.001$; Figure 2-3b), but there was no difference in fat mass between the groups ($F_{1,43.3} = 0.02$, $P = 0.89$; Figure 2-3c). As during the treatment period, males had more lean mass than females ($F_{1,27.3} = 13.93$, $P < 0.001$), but females on average had more fat than males ($F_{1,40.4} = 6.30$, $P = 0.02$). Birds with longer tarsus had more lean mass ($F_{1,28.5} = 49.59$, $P < 0.00001$). Tarsus was not a significant covariate for fat mass ($F_{1,44.6} = 0.16$, $P = 0.69$), but birds with more lean mass had more fat mass ($F_{1,66.9} = 7.90$, $P < 0.01$). There was no significant effect of age in either model assessing fat or lean mass (Fat: $F_{1,35.9} = 2.36$, $P = 0.13$; Lean: $F_{1,39.0} = 2.77$, $P = 0.10$). None of the higher order interactions between sex, treatment and age were significant in the lean or fat mass model (all $P$s > 0.25). Nest of origin did remain in the model assessing lean mass (Wald $Z = 1.90$, $P = 0.06$), but was non-significant in the fat mass model.

### 2.3.3 Testis volume

There was no significant difference in testis volume between males from either developmental treatment ($F_{1,17} = 0.36$, $P = 0.56$), participation in the cognitive experiments ($F_{1,17} = 0.69$, $P = 0.42$), or their interaction ($F_{1,17} = 0.88$, $P = 0.36$). Body mass was not a significant covariate ($F_{1,17} = 2.45$, $P = 0.14$).
2.3.4 GnRH challenge

2.3.4.1 Males

For baseline androgen levels, control males had higher baseline androgen levels than treatment males ($F_{1,15} = 5.15, P = 0.038$; Figure 2-4a). There was no difference between treatment groups at peak or integrated androgen levels (Peak: $F_{1,17} = 0.61, P = 0.44$; Integrated: $F_{1,17} = 1.12, P = 0.31$; Figure 2-4a). There was no difference between males that participated in the cognitive experiments compared to those who did not for any of the androgen measures (Baseline: $F_{1,17} = 0.29, P = 0.60$; Peak: $F_{1,17} = 0.47, P = 0.50$; Integrated: $F_{1,17} = 2.03, P = 0.17$; Figure 2-4b), nor did it interact with developmental treatment on any of the androgen measures (all $Ps > 0.24$). Adult body mass and accelerated growth were not significant covariates in any of the models (all $Ps > 0.06$, all $Ps > 0.32$, respectively).

2.3.4.2 Females

There was no difference between treatment groups for any of the androgen measures (Baseline: $F_{1,20} = 1.97, P = 0.18$; Peak: $F_{1,20} = 1.15, P = 0.30$; Integrated: $F_{1,20} = 0.004, P = 0.95$; Figure 2-5a). However, females that participated in the cognitive experiments had significantly higher baseline androgen levels ($F_{1,18} = 7.81, P = 0.012$; Figure 2-5b), but it was not a significant variable for peak or integrated androgen levels (Peak: $F_{1,20} = 0.23, P = 0.64$; Integrated: $F_{1,20} = 0.002, P = 0.96$; Figure 2-5b). There were no significant interactions between developmental treatment and participation in the cognitive experiments (all $Ps > 0.28$) and adult body mass was not a significant covariate in any of the models (all $Ps > 0.15$).
Figure 2-4 Effects of (a) developmental treatment (control versus treatment) and (b) participation in the cognitive experiments (yes versus no) on male plasma androgen levels during baseline sample, peak (30 minute sample – baseline sample), and integrated measures (area under the curve). All data were log transformed to meet assumptions of normality. Bars represent means ± SE. * $P < 0.05$. 

A

B
Figure 2-5 Effects of developmental treatment (control versus treatment) and (b) participation in the cognitive experiments (yes versus no) on female plasma androgen levels during baseline sample, peak (30 minute sample – baseline sample), and integrated measures (area under the curve). All data were log transformed to meet assumptions of normality. Bars represent means ± SE. * P < 0.05.
Accelerated growth rate remained in the final model for integrated androgen levels, although it was marginally non-significant ($F_{1,18} = 3.88, P = 0.06$; Figure 2-6). Females that grew faster in the ten days following the food-restriction treatment had lower integrated androgen levels. Accelerated growth was not a significant covariate in the models for baseline ($F_{1,20} = 0.29, P = 0.59$) or peak androgen levels ($F_{1,20} = 1.01, P = 0.33$).

Figure 2-6 Relationship in females between growth rate 10 days post-treatment and integrated androgen levels in adulthood. Females with higher growth rates had lower integrated androgen scores in adulthood.

### 2.4 Discussion

Unpredictable food conditions in development affected body composition during the treatment, growth rates following the treatment period (i.e., accelerated growth), and aspects of HPG function in both sexes. Overall, during the developmental treatment growth rate as measured by body mass did not differ between groups, but treatment birds had less body fat during the treatment period. As predicted, treatment birds compensated...
once the restriction was lifted and this was due to the allocation of a resources towards somatic growth (i.e., lean mass) rather than storing the energy surplus as fat. This suggests that accelerating growth was a strategy used by treatment birds to mitigate the adverse effects of chronic food unpredictability during the juvenile period (Metcalf and Monaghan 2001). Yet, in adulthood there was no difference in body mass between the developmental treatments. The differences in resource allocation during the juvenile period had long-term effects on the regulation of androgens in males. In adulthood, treatment males had lower basal androgen levels than control males. In females, food-restriction in the later yearling period (as a function of the cognitive experiments in Chapter 3) elevated basal androgen levels. Moreover, females who gained the most mass following the termination of the developmental treatment showed the largest reduction in integrated androgen levels in adulthood. This relationship suggests increased mass may have incurred a cost by reduced function of the HPG axis.

2.4.1 Growth rates

Food unpredictability typically reduces developmental growth rates (Brumm et al., 2009; Nowicki et al., 2002; Schmidt et al., 2012), but that was not observed here. Although I used the same unpredictable food protocol, treatment birds from Buchanan et al. (2003) weighed more than control birds during the treatment period. A point of difference was that my birds were individually housed, while the starlings from Buchanan et al. (2003) were housed socially. Socially housing starlings is known to cause increases in body mass, compared to birds that live individually, as birds in groups may put on mass as a strategy to aid in competing for resources (Witter and Goldsmith 1997).
Future studies should consider how the social environment interacts with developmental stressors to affect various phenotypic outcomes.

Accelerated growth, a typical pattern in this species (Farrell et al. 2011) and others (Fisher et al. 2006; Schmidt et al. 2012; Kriengwatana et al. 2013), was observed following the termination of the treatment. Accelerated growth suggests that food unpredictability delayed the development of various physiological systems (such as the HPG axis studied here). Subsequently, birds compensated by maximizing, rather than optimizing, growth rates to recuperate slowed development (Metcalfé and Monaghan 2001). This was however a short-term strategy as there was no measurable difference in adulthood between birds from either treatment. The observed sex difference in adult body mass was likely due to the fact females were in breeding condition and weigh more than males on account of the development of the ovary and oviduct (Ricklefs and Hussell 1984; Meijer et al. 1994). Interestingly, birds that were subjected to food-restriction by way of participating in the cognitive experiments weighed significantly more than birds that did not experience this restriction. After enduring several months of food-restriction, birds may have altered their food intake and/or metabolic activity as a measure to prepare for future food unpredictability. Unfortunately, I do not know if increased body mass was primarily due to changes in lean and/or fat mass as the QMR was unavailable during the adult analysis period. I also did not monitor food intake, therefore I can not attribute this increase in body mass to increased food intake and/or changes to metabolism, or behaviour which resulted in lower energy consumption (Fokidis et al. 2012). Including food intake and measures of behavioural activity in future food-restriction studies is necessary to distinguish between these competing explanations.
2.4.2 Body composition

Overall, variation in body mass and fat followed the typical developmental trajectory observed for juvenile starlings (Kessel 1957; Meijer et al. 1994). Fat reserves peaked at approximately 45-55 days of age, which coincides with the onset of the first prebasic moult (Kessel 1957). During the treatment period, birds in both groups followed similar developmental trajectories for fat, but treatment birds had less fat mass than control birds overall. This suggests control birds had an energy surplus that they could store as fat mass (Ricklefs et al. 1998). Control birds may have had more resources available to divert towards the development of physiological systems, such as the HPG axis. Within these same birds, other deficits have been observed on behavioural and neural measures (Chapter 3-5; Farrell et al. 2015a; Farrell et al. 2015b). Coupled with the data from this study, these findings suggest that unpredictable food conditions in early life affect the development of multiple physiological, specifically neural, systems and have long-term effects on the adult phenotype.

One adaptive strategy for animals encountering periods of food scarcity is to increase fat stores (Ricklefs et al. 1998; Schew and Ricklefs 1998). A previous study in juvenile starlings that removed their food every other day, at the same time of day, gained more weight and body fat compared to juveniles that had ad libitum food access (Witter and Swaddle 1997). A key difference between this study and mine is that the food-restriction was predictable, and therefore birds could strategically adjust their food intake, or metabolic requirements, to ensure they had enough resources to sustain them through suboptimal conditions (Witter et al. 1995). When food access is unpredictable for extended periods of time this can induce a chronic stress response by increasing
circulating glucocorticoids (Love and Williams 2008; Schoech et al. 2009; Kriengwatana et al. 2014). In curve-billed thrashers (*Toxostoma curvirostre*), unpredictable access to food, not differences in energy consumption, caused changes in metabolism and hormones that resulted in maladaptive use of energy reserves (i.e., reductions in body weight and fat mass) and increased metabolic activity (Fokidis et al. 2012). Although I did not measure glucocorticoids, starlings raised on a similar protocol exhibited a greater glucocorticoid capture and restraint response than controls (Buchanan et al. 2003). Glucocorticoids have lipolytic properties (Campbell et al. 2011) and if food-restricted birds have higher glucocorticoids, this may have facilitated fat loss through lipolysis.

When food availability was unpredictable, treatment birds may have allocated resources preferentially to maintain the growth of critical systems by slowing the growth of others (Schew and Ricklefs 1998; Brzek and Konarzewski 2004). Following the treatment period, treatment birds gained substantially more lean mass than control birds, which suggests lean tissues were immature and birds increased the allocation of resources to the development of organs and/or muscles. Lean tissue decreases its water content as its growth rate slows (Ricklefs and Webb 1985), thus a sign of tissue immaturity is increased water content (Killpack et al. 2014). QMR is only able to estimate wet lean mass. Wet lean mass encompasses dry lean mass, and although they are correlated within an individual (Ardia 2005), without performing carcass analyses we were not able to differentiate wet/dry lean mass components by tissue source to determine which source of lean tissue was affected most by the developmental treatment. Accelerated development of total lean mass as measured by QMR may be influenced the greatest by large sources of lean mass, which for developing birds include the pectoralis muscles and the digestive
organs (Ricklefs et al. 1998; Brzek and Konarzewski 2001). Young songbirds developing on a food-restriction protocol exhibited compensatory growth of the pectoralis muscle and the digestive organs once the food-restriction was terminated (Brzek and Konarzewski 2004). Similar food-restriction protocols in poultry are known to cause reduction in breast muscles (i.e., pectoralis muscle), but this tissue shows remarkable compensatory growth once ad libitum nutrition is restored (Zhan et al. 2007). While both a difference in tissue maturity and/or tissue growth could be responsible for the accelerated growth observed here, to distinguish between these two explanations, carcass analyses would be necessary in future studies.

Although there was no sex difference in fat mass during the treatment period, females gained more fat after the food-restriction ended than males. At this stage, birds were finishing the first prebasic moult (Feare 1984) and early autumn is characterized by gains in fat and overall mass equally for both sexes (Meijer et al. 1994). If there was a sex difference in moult duration this could explain this result. I did not monitor moult progress for birds individually, but there was tremendous variation in moult duration among individuals (personal observation). It is possible that if females completed moult faster than males they may have begun the typical autumnal fat storing sooner.

2.4.3 HPG axis

2.4.3.1 Males

Developing in an environment with unpredictable food affected male HPG function into adulthood. Control males had higher baseline androgen levels than treatment males. In adulthood, chronic stress activates the HPA axis, and increased glucocorticoids inhibit HPG axis functioning (Tsigos et al. 1999; Kyrou and Tsigos
Circulating levels of androgens could be higher in the control males because their testes may be synthesizing more T and DHT. In starlings, increases in T during the breeding season are associated with increases in song rate and syrinx size, which may modulate the quality of singing (Ball and Balthazart 2010). Although the difference in baseline androgen levels between the treatment groups was small (~1ng/mL), control males may have been able to afford the costs of higher circulating androgens to reap the benefits for their courtship song.

In mammals, increased glucocorticoid exposure can result in apoptosis of Leydig cells (primary site of testosterone production in the testis) and may cause long-term reductions in testosterone synthesis (Hardy et al. 2005). Additionally, increased androgens in control males may be due to effects further upstream. Control males may have had a greater number of neurons expressing GnRH, which could cause higher testicular testosterone synthesis (Dawson et al. 2001). My results are in accordance with the mammalian literature, but in contrast with the results from the only songbird study to date. Schmidt et al. (2014) found that circulating androgen levels were higher in male song sparrows that had been given glucocorticoid treatment in development, but males reared in unpredictable food conditions did not differ from control males. Discrepancies between these studies could be a function of species differences, form of developmental stress (glucocorticoid treatment versus food supply manipulations), the intensity of how the developmental stressor was enforced, or some combination of these factors. Future research in how early developmental conditions affect the development of the HPG axis should incorporate measures of GnRH neuronal plasticity and/or Leydig cell
development to determine how different environmental stressors affect the function of the HPG axis.

2.4.3.2 Females

Chronic food-restriction exerted opposing effects on HPG function if experienced in early versus later development. Food unpredictability in development did not directly affect androgen production in females. However accelerated growth was correlated with integrated androgen levels: females that grew more rapidly following the end of the developmental treatment had lower androgen production. But, chronic food-restriction prior to the breeding season increased baseline levels of androgens. The theca interna cells of the ovary produce androgens and when produced in excess androgens enter into general circulation to exert effects on physiology and behaviour (Nelson 2011). However, most androgens will be aromatized into estradiol, which is associated with female reproductive behaviours (Searcy and Marler 1981; Christians and Williams 1999). Therefore, androgens may exert their effects directly, or indirectly further downstream through estrogen synthesis. The HPG axis is one system that was developmentally traded off to facilitate accelerated body growth, which could have long-term adverse consequences on a female’s future reproductive behaviour and breeding success (Rutkowska et al. 2005; López-Rull and Gil 2009).

Chronic food-restriction experienced prior to the breeding season may have worsened female’s condition. To compensate for being in poor condition, females may have increased androgens to help modulate behaviours that would increase her ability to compete with females in better condition Increased testosterone in reproductive females can increase intrasexual aggression (Zysling et al. 2006; Sandell 2007) and enhance
acquisition and maintenance of a breeding site (Veiga and Polo 2008). In starlings, increased female-female aggression establishes a monogamous pair bond and ensures that a male partner does not give resources to another female (Sandell 1998). However, increased levels of T are also associated with decreased immune function (Zysling et al. 2006), reduced mate choosiness (McGlothlin et al. 2004), delayed onset of reproduction (Clotfelter et al. 2004), reduced breeding success (López-Rull and Gil 2009), and reduced fecundity (Rutkowska et al. 2005). Environmental stressors may mediate the maternal phenotype and cause females in poor condition to engage in a strategy which could maximize current reproductive success, at the expense of future reproductive success, by mediating androgen regulation (Veiga and Polo 2008; Cain and Ketterson 2013).

2.4.4 Conclusions

Stressful rearing environments in the juvenile period may not exert drastic changes to overall body growth, but subtle changes in body composition (i.e., less fat mass) may be indicative of strategies to cope with an unpredictable food supply. Once ad libitum access to food had been restored, birds compensated by gaining lean mass, which suggests that food-restriction delayed lean tissue maturation and/or growth of lean tissue. Developmental trajectories and differences in body composition during the juvenile period were associated with long-term effects on the HPG axis in adulthood. Baseline androgen levels of adult males were lowered by early food-restriction and females that maximized growth after the food-restriction ended may have incurred a cost of reduced HPG axis function as measured by lower integrated androgen levels. Declines in androgens could have adverse effects on mating and reproductive success for both sexes. However, females that experienced stress preceding the breeding season (as a function of
participating in the cognitive experiments) had higher baseline levels of androgens. This study highlights how monitoring growth and body composition changes can be markers for future endocrine regulation associated with future reproduction and other adult outcomes (Chapters 3-5; Farrell et al. 2015a; Farrell et al. 2015b).
2.5 References


West BT, Welch KB, Galecki AT (2007) Linear mixed models: a practical guide to using statistical software. Taylor and Francis Group, Boca Raton (FL)
Chapter 3

3 Developmental stress impairs performance on an association task in male and female songbirds, but impairs auditory learning in females only

3.1 Introduction

Birdsong is a complex learned vocalization that has evolved within the context of communication networks of signalers and receivers (Searcy and Nowicki 2005). Predominantly males sing, using their songs to attract mates and/or defend territories (Catchpole and Slater 2008). Comparatively, females of most species sing less than males, but assess song quality and preferably mate with singers that excel at producing certain song features (i.e., song complexity, local dialects, song output) as these traits often correlate to aspects of male quality (Nowicki and Searcy 2005). The quality of song varies tremendously between conspecifics and this phenomenon has been explained in part by the developmental stress hypothesis (Nowicki et al. 1998; Nowicki et al. 2002; Spencer and MacDougall-Shackleton 2011; MacDougall-Shackleton and Spencer 2012). In brief, males reared in stressful environments are unable to allocate as many resources to developing the costly neural structures (i.e., the song-control system) that enable song learning and production, which ultimately affects the quality of the adult song phenotype. Therefore, song is reflective of early developmental conditions, with only males of superior phenotypic and/or genotypic quality being able to produce high quality song.

Developmental stress may also affect perception and/or discrimination of song. Song production and perception are regulated by different neural circuits, but are
intricately linked (Woolley and Moore 2011; Lynch et al. 2013). To date, female song preferences have been the metric used to assess the effects of developmental stress on song discrimination (Woodgate et al. 2010; Woodgate et al. 2011; Schmidt et al. 2013a; Farrell et al. 2015c). The results have been mixed—some studies have found that developmental stress weakens song preferences (Schmidt et al. 2013a; Farrell et al. 2015c) or reduces activity levels in mate choice trials (Woodgate et al. 2010), but other studies have found no change in the direction or strength of song preferences (Woodgate et al. 2010, 2011). Nevertheless, preference is not purely a measure of discrimination and is influenced by several factors (e.g., self referential effects, motivation, number or quality of males available, environmental factors; Cotton et al., 2006). Researchers have to account for these factors first before explaining weaker song preferences by a reduction in perceptual or cognitive abilities necessary for trait discrimination. Furthermore, song preferences are a female biased metric. Although males in some species show song preferences (zebra finches, *Taeniopygia guttata*: Riebel et al. 2002), in others they do not (European starlings, *Sturnus vulgaris*: Gentner and Hulse 2000). The most direct method to assess how developmental stress affects auditory discrimination in both sexes is to assess discrimination of auditory stimuli independent of mate preference paradigms.

Songbirds excel at auditory tasks assessing frequency perception, and sound frequency is a defining feature for conspecific song recognition (Weary et al. 1986; Nelson 1989). If developmental stress affects frequency discrimination, this could cause adverse effects on song learning and weaken female song preferences. A variety of operant-conditioning assays have been developed that suggest songbirds extract two main dimensions of frequency information: absolute frequency and relative frequency.
(Weisman et al. 1994; Weisman et al. 1998). Absolute frequency discrimination is the ability to determine the frequency of a sound with no external referent, while relative frequency discrimination is the ability to encode frequency information of sounds in relation to each other. Several species have demonstrated impressive abilities to learn and categorize novel stimuli based on absolute, relative, or both forms frequency information (Hulse et al. 1990; Weisman et al. 1994; MacDougall-Shackleton et al. 1998; Weisman et al. 1998; Lee et al. 2006). Operant conditioning paradigms can provide a powerful behavioural assessment of trait discrimination, independent of song preferences, and can be used to evaluate both sexes.

Although these tasks can assess auditory discrimination, performance can be influenced by additional cognitive processes, such as behavioural flexibility and inhibitory control (Coppens et al. 2010). Various protocols to assess cognitive abilities have been developed for captive songbirds (e.g., Boogert et al. 2011; Amy et al. 2012) and have been successfully implemented in studies examining the effects of developmental stress on learning impairments into adulthood (Fisher et al. 2006; Kriengwatana et al. 2015). It would be prudent to conduct additional cognitive tests for two reasons. First, any observable impairment in performance on auditory tasks may be due to an auditory-specific learning deficit, or a global learning deficit. Performance across various tasks could be used to assess if developmental stress affects auditory processes specifically. Secondly, some studies have sought to determine if songbirds possess general cognitive abilities. Positive relationships have been noted between male song quality and cognitive performance (Boogert et al. 2008; Boogert et al. 2011; Farrell et al. 2011), but several studies have found negative or null relationships (Boogert et al.
Female songbirds are typically not considered in studies assessing correlated cognitive traits because song quality, a male-typical behaviour, is often the primary trait to which all other cognitive abilities are compared. Auditory discrimination abilities are needed both for song learning and trait discrimination. Assessing auditory discrimination, instead of song quality, in conjunction with other cognitive tests (e.g., inhibitory control, association learning) allow us to include females into the evaluation of whether developmental stress affects various cognitive processes equally (Farrell et al. 2015a).

In the present study, I tested the effects of a developmental stressor on auditory discrimination in starlings. I manipulated early-developmental conditions through an unpredictable food supply treatment. After the developmental treatment ceased, I used an operant conditioning paradigm to assess two important aspects of auditory discrimination: (1) absolute frequency discrimination and (2) relative frequency discrimination. After auditory testing, I assessed associative learning in two tasks using non-auditory stimuli (i.e., colour) to determine if developmental stress caused broad impairments to association learning and inhibitory control. I compared learning across auditory and visual tasks to assess if developmental stress impairs learning processes broadly, or selectively affects learning within particular modalities. I hypothesized that birds of both sexes raised in stressful early-life environments (i.e., the treatment group) would exhibit poorer performance on auditory and colour association tasks compared to their control counterparts. However, I had no a priori reason to assume that the learning processes that support auditory learning are the same as those that support colour
association learning. Therefore, I hypothesize performance on the auditory and visual tasks will not be correlated.

3.2 Methods

3.2.1 Subjects

The birds used in this study were a subset of the same birds described in Chapter 2 (Farrell et al. 2015b). Throughout the duration of the experiment, 9 adult starlings (male: 5, female: 4) were housed in the center of the colony room and served as song tutors for the duration of the experiment. Adult birds were caught from a farm around London, Ontario in 2008.

3.2.2 Auditory discrimination

I assessed how early developmental stress affected absolute and relative frequency auditory discriminations using tasks modeled after studies conducted with other songbird species (Njegovan and Weisman 1997; Sturdy et al. 2001). All birds completed the absolute frequency task first, then the relative frequency task. At 6 months of age, I trained a randomly selected subset of birds (n = 23; Table 1) to use the operant apparatus and testing concluded when birds were 10-12 months of age. I tested birds daily between 0900-1700 h, rotating birds every 2 hours. Therefore, each bird was tested

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sex</th>
<th>Sample Size</th>
<th>Completed Auditory</th>
<th>Completed Colour</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>Male</td>
<td>9</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>9</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Treatment</td>
<td>Male</td>
<td>8</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>12</td>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>
consistently at the same time of day. As both tasks used food as a reward, I employed a restricted feeding schedule to ensure that motivation during testing was high. I weighed subjects prior to and after their testing session. Based on these measurements, I allocated the appropriate amount of food to maintain each subject at 85-90% of their free feeding weight. Throughout the auditory tasks, I maintained a constant long day photoperiod (LD 15.5:8.5).

3.2.2.1 Operant apparatus

During the experiments, starlings were housed in a modular test chamber (30 x 24 x 29 cm; Med Associates Inc., St Albans, Vermont, U.S.A.) containing a response panel, food hopper, house light, and speaker. The test chamber was inside a custom made sound attenuating chamber (107 x 48 x 46 cm) lined with sound attenuating material (Noise-Blok, Wilrep Ltd, Mississauga, Ontario, Canada). The response panel contained two circular response keys (diameter 2.5 cm) located in a horizontal line 4 cm above and on the either side of the opening to the food hopper (5 x 6 cm). The response keys were attached to a microcontroller (8 Input, 16 Output SmartCtrl, Med Associates Inc.) and computer running MED-PC IV Software (Med Associates Inc.), which controlled input and data output. Auditory stimuli were played over a computer speaker (Logitech s11; Freemont, CA, U.S.A.) connected to the computer. Water was available at all times through a bottle attached on the wall opposite the food hopper.

3.2.2.2 Training procedures

Shaping

Once birds were habituated to the chamber, I trained them to peck the response keys by taping a few grains of chick starter to them. First, pecks to either the left or right
response key would give the bird 3 s access to the food hopper. Following this, birds had to peck the left key followed by the right key to gain access to food. Once birds were pecking at a high and constant rate (pecks within a 2 h session $\geq 250$), nondifferential training began the next day.

*Nondifferential training*

Each task was preceded by at least three days of nondifferential training, or until the bird responded to more than 80% of trials with no significant difference between the category of stimuli (assessed via $t$-test). The objective was to ensure that responding was uniform across all stimuli and that a bird had no inherent auditory biases prior to testing.

Within a trial, a bird would peck the left key. This would cause an auditory stimulus from the task to play at random. After 1 s from the stimulus onset, the right response key would be active for a 4 s response window. The response key was delayed by 1 s to ensure that the bird listened to the entirety of the stimulus before responding. A bird was rewarded with 3 s access to food if they pecked the response key within the response window. However, if the bird did not peck the right key within the response window the trial would end. In either outcome, a 3 s intertrial interval would follow.

*Discrimination training*

Pecking the right key in response to a rewarded (S+) auditory stimulus was still rewarded with 3 s access to food. However, pecking the right key in response to an unrewarded (S-) auditory stimulus resulted in a 25 s light-outs phase. A 3 s intertrial interval would follow either outcome. The birds were tested daily in 2 h sessions, or until they completed the maximum number of trials allowed per day (experiment 1: 500, experiment 2: 600).
3.2.2.3 Experiment 1: absolute frequency

I synthesized 40 individual tones that ranged in frequency from 980-5660 Hz, each tone rising in 120 Hz increments. All tones were created using Audacity software (version 2.0.2; D Mazzoni; http://audacity.sourceforge.net) with output settings 44100 kHz, 32-bit samples/s. Each tone was 440 ms in duration and the 5 ms onset and offset for each tone was tapered in amplitude to remove transients. I created two versions of each tone to control for possible amplitude cues. On alternating training days, I played either a 65 dB (SPL) or 75 dB (SPL) version of the tone as measured with a sound meter inside the operant apparatus. The 40 tones were divided into 8 series, wherein each series comprised 5 successive tones. Series alternated between tones that were rewarded and unrewarded (Table 2). Therefore, there were 20 S+ tones (series 1, 3, 5, and 7) and 20 S- tones (series 2, 4, 6 and 8). Training continued on the absolute frequency task until birds achieved a predetermined performance criterion (explained below).

Table 3-2 Frequencies (Hz) of the rewarded (S+) and unrewarded (S-) series of tones in the absolute frequency discrimination

<table>
<thead>
<tr>
<th>Series</th>
<th>Reinforcement Status</th>
<th>Frequency (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S+</td>
<td>980 1100 1220 1340 1460</td>
</tr>
<tr>
<td>2</td>
<td>S-</td>
<td>1580 1700 1820 1940 2060</td>
</tr>
<tr>
<td>3</td>
<td>S+</td>
<td>2180 2300 2420 2540 2660</td>
</tr>
<tr>
<td>4</td>
<td>S-</td>
<td>2780 2900 3020 3140 3260</td>
</tr>
<tr>
<td>5</td>
<td>S+</td>
<td>3380 3500 3620 3740 3860</td>
</tr>
<tr>
<td>6</td>
<td>S-</td>
<td>3980 4100 4220 4340 4460</td>
</tr>
<tr>
<td>7</td>
<td>S+</td>
<td>4580 4700 4820 4940 5060</td>
</tr>
<tr>
<td>8</td>
<td>S-</td>
<td>5180 5300 5420 5540 5660</td>
</tr>
</tbody>
</table>

Response Measures

I calculated a discrimination ratio (DR) to assess each bird’s daily overall discrimination. To calculate the DR, I divided the number of responses to rewarded
stimuli (S+) by the total number of responses (to both rewarded and unrewarded stimuli). In signal detection terminology, this is the number of correct hits divided by the sum of correct hits and false alarms. Therefore, a DR of 0.50 is equivalent to chance levels of discrimination (responding equally to rewarded and unrewarded stimuli), whereas as a DR of 1.00 was a perfect level of discrimination (all responses are to the rewarded stimuli only). Testing continued until a bird achieved a DR ≥ 0.80 for three consecutive days.

While the DR provides a way to assess selective responding to groups of stimuli, it does not provide information about accuracy of responding to individual stimuli. It is possible to achieve a high DR while not being very accurate at the discrimination, such as when a bird responds only to a select few rewarded stimuli and withholds responding to any of the unrewarded stimuli. To evaluate how well each individual stimulus was discriminated, I adapted a two-tailed 95% confidence interval (CI) approach used by others (Weisman et al. 1994; Sturdy et al. 2001) to the final three days of testing when birds were performing at a criterion of a DR ≥ 0.80 (i.e., asymptotic performance). In brief, for each bird I calculated the 95% CI from the mean and standard deviation to all the unrewarded stimuli. I then calculated the mean percentage response for each individual rewarded stimulus and compared it to the 95% CI for the unrewarded stimuli. If the percent responding to a rewarded stimulus fell within the 95% CI for the unrewarded stimuli then this would be statistical evidence that the bird did not discriminate that stimulus as different from the unrewarded ones. Therefore, for each rewarded stimulus outside the 95% CI this was statistical validation for that particular stimulus being discriminated at a level of α < 0.05. I tallied the total number of stimuli that each bird discriminated above the 95% CI.
3.2.2.4 Experiment 2: relative frequency

I synthesized pairs of tones following the same sampling rates, tone duration, tapering, and amplitude parameters as outlined in the absolute frequency discrimination. I created tone pairs by separating each tone by a 100 ms silence. Again, to control for amplitude cues I created two different amplitude versions of each tone and constructed pairs so that one of the tones was played at a higher amplitude, 75db (SPL), than the other tone, 65db (SPL). The tone that was higher in amplitude alternated across tone pairs within a session and between sessions within a tone pair.

Tones pairs were constructed following a semitone metric (see Weisman et al. 1994). In brief, the semitone metric provides a mathematical basis by which to calculate the frequency ratios. An octave is a doubling in frequency, which can be divided into 12 semitones. Therefore, the note pairs were constructed such that tones were separated by a frequency difference of zero, one, or four semitones. In total, I had three categories of note pairs and all exemplars within a category shared a common frequency ratio difference between Tone 1 and 2 (ratios used 1.00, 1.06, and 1.26). Each category contained 9 exemplars, with the rewarded category being assigned a ratio of 1.06, while the remaining 18 exemplars were in the unrewarded ratio categories 1.00 and 1.26 (Table 3). Tone pairs were created such that each stimulus contained one note whose absolute frequency was in an exemplar in at least two of the three ratio categories, and in most cases was present in all three categories. I erroneously constructed one S+ exemplar to a ratio of 1.12 instead of 1.06. However, as the nature of the task was to assess speed of task acquisition across treatment conditions, and not a psychophysical task assessing concept or rule learning at a species level, I left this exemplar in all statistical analysis as removing it did not alter any of my main findings.
Table 3-3 Frequencies (Hz) of the tone pairs used in the relative frequency discrimination.

<table>
<thead>
<tr>
<th>Note 2</th>
<th>Ratio 1.0</th>
<th>Ratio 1.06</th>
<th>Ratio 1.26</th>
</tr>
</thead>
<tbody>
<tr>
<td>2519</td>
<td>2519</td>
<td>2669</td>
<td>3178</td>
</tr>
<tr>
<td>2669</td>
<td>2669</td>
<td>3087</td>
<td>3676</td>
</tr>
<tr>
<td>2748</td>
<td>2748</td>
<td>2912</td>
<td>3469</td>
</tr>
<tr>
<td>2912</td>
<td>2912</td>
<td>3087</td>
<td>3676</td>
</tr>
<tr>
<td>3087</td>
<td>3087</td>
<td>3272</td>
<td>3897</td>
</tr>
<tr>
<td>3272</td>
<td>3272</td>
<td>3469</td>
<td>4130</td>
</tr>
<tr>
<td>3469</td>
<td>3469</td>
<td>3676</td>
<td>4371</td>
</tr>
<tr>
<td>3676</td>
<td>3676</td>
<td>3897</td>
<td></td>
</tr>
<tr>
<td>3785</td>
<td>3785</td>
<td>4012</td>
<td>4770</td>
</tr>
<tr>
<td>4012</td>
<td>4012</td>
<td>4500†</td>
<td>5055</td>
</tr>
</tbody>
</table>

† One S+ exemplar was erroneously constructed to a ratio of 1.12.

Response Measures

Compared to the absolute frequency discrimination, the relative frequency was acquired more slowly. Therefore, I trained birds for 90 days, as opposed to a predetermined performance criterion. My objective was to assess treatment effects on auditory discrimination. I ended the experiment at 90 days as there was ample variation in performance and it ensured that these experiments would be completed prior to the first breeding season, which was when the males were slated to complete a courtship song experiment (see Chapter 5).

I calculated the daily DR for all birds. I did not apply the 95% CI metric to the relative frequency results, as most birds did not achieve a DR ≥ 0.80 and therefore the 95% CI assessment is not an appropriate analysis. In its place, I calculated the average % responding to the rewarded category and the unrewarded categories for the final three days of testing. Only 7 birds (control: n = 4; treatment: n = 3) achieved levels of
asymptotic performance, thus I refer to this as an end-point measure of performance accuracy.

3.2.3 Colour discrimination

I assessed colour association learning on all birds (n = 38; Table 1) when they were 11 to 13 months of age. I modeled my task after similar protocols with captive songbirds (Boogert et al. 2011; Amy et al. 2012; Kriengwatana et al. 2015). In brief, I assessed birds’ ability to forage for mealworms concealed by different coloured tiles. Using progressive shaping, I trained birds to push aside tiles that covered the wells on the foraging tray. All testing was done in birds’ home cages daily between 0900-1300 h while birds were maintained on a short day photoperiod (10L:14D). Individual birds were tested at approximately the same time daily. Motivation throughout shaping and testing was high, as birds were held at 85-90% of their ad libitum weight in the same manner as described in the auditory tasks.

3.2.3.1 Foraging apparatus and training

The experimental tray used was a metal muffin baking pan (27 x 18 cm) with 12 wells (three rows of 4 wells; well diameter: 4.5 cm) equally spaced apart (1.5 cm). Felt covers covered opening to the wells, which were held in place by small pieces of Velcro glued around each well. A bird could only gain access to a well by pushing its beak under the cover and pushing upwards. Mealworms were hidden in wells under felt covers as the reward for this task.

Each day of shaping consisted of 6 trials. Using progressive approximation, I trained birds to remove tiles to gain access to mealworms hidden in the wells. In stage 1, the bird was presented with the tray and 2 mealworms were placed in random wells,
adjacent to which I placed a black tile. In stages 2, 3 and 4 the black tile progressively covered \( \frac{1}{4}, \frac{1}{2} \) and \( \frac{3}{4} \) of the opening of a baited well, respectively. Lastly, on stage 5 the opening of the baited well was entirely concealed by the black tile. To pass a trial, both worms had to be eaten within 5 minutes. If a bird ate only 1 of the worms, I scored this as a neutral outcome. If neither worm was eaten, the bird failed the trial. To progress to the next stage, a bird had to pass three consecutive trials. If a bird received three consecutive fails, it regressed to the previous stage. Once the bird passed the final stage of shaping it commenced the colour association learning task the following day.

### 3.2.3.2 Colour association learning task

During the test trials, only 8 of the 12 wells were covered. I covered at random 8 wells with 1 green tile and 7 orange tiles (or, 1 orange and 7 green; rewarded colour was counterbalanced across subjects). Under the uniquely coloured tile I placed a mealworm. Testing consisted of 12 trials daily and on each trial I randomly covered a new arrangement of 8 wells. On each trial, I recorded the order tiles were searched. A bird completed the task when it searched the rewarded tile first for 5 consecutive trials. Once criterion was met, testing was completed for that day and the bird commenced the inhibitory learning task the following day.

### 3.2.3.3 Inhibitory learning task

In this task, I exchanged one of the previously unrewarded tiles with a novel blue tile that now signaled the location of the mealworm. On each trial, 8 wells still remained covered. For example, there would be 1 blue tile, 1 (now unrewarded) green tile, and 6 unrewarded orange tiles. Testing proceeded in the same manner as the colour task outlined above. In addition to recording the order the tiles were searched, I also noted if
the green tile was searched first before the blue tile (a perseverative error). The criterion for passing the task was the same as above.

Response Measures

For both experiments, I calculated for each bird the total number of trials completed until criterion was achieved. I also recorded how many tiles a bird searched within a trial before locating the correct tile. Searching incorrect tiles within a trial were calculated as within-trial errors. In the inhibitory learning task, I recorded perseverative errors (as described above). I did not record the duration of trials, as most birds were highly motivated to engage with the apparatus and usually began feeding within seconds.

3.2.4 Data and statistical analysis

Performances on all tasks were analyzed using linear mixed models. I examined the effects of the developmental condition (i.e., treatment) and sex. Therefore, treatment, sex, and their interaction were entered as fixed effects in all models. Models were subsequently tailored to each experiment by adding additional discrete fixed effects of interest (plus any of their higher order interactions with treatment and sex) and continuous covariates of interest. Nest of origin was included in all models as a random effect. I constructed all models first as fully loaded models (all predictors and interactions included). Then, I calculated the minimal adequate models by sequentially removing non-significant interaction terms and assessing model fit using log-likelihood ratio tests (Chapter 2; West et al. 2007). Therefore, all results reported herein are from the minimal adequate models, using restricted maximum likelihood estimation, and all post-hoc tests were conducted with Bonferroni corrections. Any statistics reported for non-significant
terms are the values from the full model prior to removal. I checked residuals for each model against a normal distribution and found no violations of normality.

For the absolute frequency task, I ran separate models on the total number of days until criterion was met and the average DR across 3-day blocks for the first 24 days of testing (i.e., when the first bird finished the experiment). I also calculated the final percentage of rewarded stimuli that were accurately discriminated from the 95% CI for all unrewarded stimuli. There was no variation across birds and therefore I did not construct a model to analyze this variable (explained below in Results). For the relative frequency task, I ran separate models on the DR throughout the task (averaged across 10-day blocks) and then the average percentage responding to each of the 3 categories of stimuli for the final 3 days of testing (with category entered as a fixed effect). Block was entered as a repeated factor (scaled identity) and included as a fixed effect in all applicable models. Nest of origin was included in all models as a random effect. I report its statistic when it remained in the final model.

For the colour association tasks, I constructed models to analyze the trials to criterion, the number of within-trial errors, and the number of perseverative errors as the dependent variables. The task was entered as a repeated effect (diagonal covariance structure) for all models, except with the perseverative errors (as these could only occur in the inhibitory task). In addition to sex and treatment, I also included if birds completed the auditory tasks as a fixed effect, and all higher order interactions with treatment and sex. Age at the time of testing (in days) was entered as covariate into all analyses, since not all birds were the same age when testing began. Nest of origin did not contribute significantly to any of these analyses and was removed from the final models. Although
the colour of rewarded tile was not a significant factor, its inclusion did significantly
improve model fit when analyzing number of within trial errors and therefore it remained
in the final model. However, it did not remain in the final models analyzing trials to
criterion or perseverative errors.

Lastly, I performed pairwise correlations to compare how performance on the
auditory and colour tasks related to each other. Data for each of these tasks did not
always follow a normal distribution and contained some measures that follow a ratio
scale; therefore, I used non-parametric Spearman rank correlations. SPSS software (v. 21)
was used for all analyses.

3.3 Results

3.3.1 Pre-testing training

Shaping and training of the operant apparatus was completed within 18 to 38 days
(mean ± SD = 31.5 ± 5.6 days). All birds were pecking at a high rate prior to beginning
nondifferential training (354.3 ± 75.2 pecks per session). Almost all birds completed
three days of nondifferential training (3.2 ± 0.6 days) prior to commencing testing.
Training duration did not significantly differ between treatment groups (\(F_{1,14.25} = 0.24, P
= 0.63\)) or sexes (\(F_{1,14.18} = 0.64, P = 0.44\)).

3.3.2 Absolute frequency task

All birds achieved criterion on the absolute frequency task within 24 to 81 days
(42.2 ± 20.1 days), except for one treatment female I assigned a ceiling value of 90 days.
Developmental treatment did not affect the number of days to achieve criterion
(Treatment: \(F_{1,20} = 1.79, P = 0.20\)). Females did take longer to achieve criterion than
males (Sex: $F_{1,20} = 6.75$, $P = 0.017$; males: 31.9 days $\pm$ 5.4 SE; females: 51.2 days $\pm$ 5.1 SE). Treatment females on average took the longest to reach criterion, however the sex by treatment interaction was non-significant ($F_{1,16.7} = 3.66$, $P = 0.073$; Figure 3-1a). Nest of origin did remain in the final model (Wald $Z = 1.788$, $P = 0.074$).

There was no difference in acquisition rates between the treatment groups (Treatment: $F_{1,149.7} = 0.06$, $P = 0.81$). However, treatment females acquired the task slower than treatment males (sex $\times$ treatment: $F_{1,155.43} = 4.44$, $P = 0.037$; post hoc test: $P = 0.014$; Figure 3-1b). While there was no difference in acquisition rate between the sexes ($F_{1,149.34} = 1.83$, $P = 0.18$), in the final 2 blocks males had significantly higher DRs than females (sex $\times$ block: $F_{7,154.38} = 2.35$, $P = 0.026$; post-hoc tests, block 7: $P = 0.009$; block 8: $P = 0.002$).

All birds responded to 100% of the S+ stimuli distinctly from the S- stimuli, as measured by the 95% CIs, suggesting that there were no perceptual deficits as detected by this task.

3.3.3 Relative frequency task

Birds reared in the control group acquired the task faster than birds reared in the treatment group ($F_{1,193.59} = 29.80$, $P < 0.00001$) and females acquired the task faster than males ($F_{1,193.68} = 25.86$, $P < 0.00001$). However, these significant main effects were due to control females outperforming all other birds (Sex $\times$ Treatment: $F_{1,192.94} = 14.62$, $P < 0.001$; Figure 3-2). Nest of origin was a significant random effect (Wald $Z = 2.12$, $P = 0.034$).

Birds from either treatment group did not differ in their end point performance ($F_{1,63.2} = 3.006$, $P = 0.088$), Females responded to fewer stimuli overall, but this was
Figure 3-1 Performance on the absolute frequency task by treatment (Cont: control; Trt: treatment) and sex (F: female; M: male). (A) Box plots illustrating the number of days it took for birds from each sex by treatment combination to achieve criterion performance (whiskers: minimum to maximum values; quantiles: 25th percentile, the median, and 75th percentile). (B) Acquisition curves by treatment and sex until the first bird achieved criterion performance at 24 days. Data were analyzed in 3-day blocks. All data points for each treatment by sex group have been offset to the right to prevent overlap of data points.
Figure 3-2 Performance on the relative frequency task by treatment (Cont: control; Trt: treatment) and sex (F: female; M: male) in 10-day blocks. All data points for each treatment by sex group have been offset to the right to prevent overlap of data points.

caused by females responding less to unrewarded stimuli (sex: $F_{1,63.3} = 9.21, P < 0.01$).

Birds did not respond to all ratios equally (category of stimuli: $F_{2,48.10} = 96.94, P < 0.00001$). Birds inhibited responding to unrewarded ratio 1.26 more compared to unrewarded ratio 1.0 (post-hoc: $P < 0.00001$; Ratio 1.26: 20.4 % responding ± 5.59 SE; Ratio 1.0: 57.7 % responding ± 5.59 SE).

### 3.3.4 Colour association tasks

All birds participated in the colour association tasks, except for two males (control: $n = 1$; treatment: $n = 1$). Birds completed shaping within 3 to 8 days ($3.8 ± 1.3$ SD). There was no difference in the duration of shaping between treatment conditions ($F_{1,25.6} = 0.03, P = 0.87$), sex ($F_{1,30.8} = 0.15, P = 0.70$), or their interaction ($F_{1,24.3} = 2.13, P = 0.16$). Birds completed the experiments within 3 to 8 days ($4.5 ± 1.2$ SD). Birds achieved criterion on the colour association task in fewer trials than the inhibitory
learning task (Task: F_{1,45.0} = 35.88, P < 0.0001; association learning: 17.3 trials ± 0.97 SE; inhibitory learning: 27.6 trials ± 1.62 SE; Figure 3-3).

Figure 3-3 Learning performance on the two colour association tasks by treatment (Cont: control; Trt: treatment) and sex (F: female; M: male) as measured by the number of trials until criterion performance was achieved.

Trials to criterion on either task did not differ significantly between treatment groups (F_{1,48.7} = 1.82, P = 0.18) or the sexes (F_{1,48.3} = 0.202, P = 0.66). Performance was not affected by the age of birds (F_{1,47.2} = 0.19, P = 0.67) or previous participation in the operant conditioning tasks (F_{1,34.3} = 0.03, P = 0.86). However, treatment birds committed more within-trial errors than control birds across both tasks (Treatment: F_{1,62.0} = 16.24, P < 0.001; Figure 3-4a). When considering only preservative errors, there was no difference between the treatment groups (Treatment: F_{1,27.9} = 0.021, P = 0.89; Figure 3-4b). No other variables, or their interactions, explained performance on the colour association tasks (all P-values > 0.09).
3.3.5 Comparison across auditory and visual tasks

I assessed if the learning processes that support song learning are indicative of learning in other cognitive domains. For each task conducted, I chose one dependent variable that best represented task aptitude. Then, I ran Spearman rank correlations to
assess the relationship between performance (dependent variable used in parentheses) across the absolute frequency task (days to criterion), relative frequency task (average DR on the final 3 days of testing), colour association task (trials to criterion), and colour inhibitory task (trials to criterion), the results of which are summarized in Table 4. I ran comparisons for the 22 birds that completed all 4 tasks, but I also did a separate analysis for all birds that completed both of the colour tasks (values in parentheses in Table 4). I found no significant relationships between learning performances on any of the tasks (all P-values > 0.37; Table 4).

Table 3-4 Spearman correlation matrix of performance across both auditory tasks (absolute frequency and relative frequency) and colour association tasks (colour association and colour inhibitory)

<table>
<thead>
<tr>
<th>Absolute Frequency</th>
<th>Relative Frequency</th>
<th>Colour Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative Frequency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r_s$</td>
<td>-0.200</td>
<td>0.094</td>
</tr>
<tr>
<td>$P$</td>
<td>0.372</td>
<td>0.780</td>
</tr>
<tr>
<td>$N$</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Colour Association</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r_s$</td>
<td>-0.063</td>
<td>0.058</td>
</tr>
<tr>
<td>$P$</td>
<td>0.780</td>
<td>0.797</td>
</tr>
<tr>
<td>$N$</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Colour Inhibitory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r_s$</td>
<td>0.111</td>
<td>-0.113 (0.160)</td>
</tr>
<tr>
<td>$P$</td>
<td>0.621</td>
<td>0.617 (0.350)</td>
</tr>
<tr>
<td>$N$</td>
<td>22</td>
<td>22 (36)</td>
</tr>
</tbody>
</table>

Values in parentheses include subjects that only completed the colour association tasks.

3.4 Discussion

A stressful early-life environment selectively affected the learning on both auditory discrimination tasks for female starlings, but not for males. However, developmental stress did not permanently impair auditory perceptual accuracy: all birds
achieved perfect accuracy when criterion performance was assessed on the absolute frequency task. In comparison, developmental stress negatively affected performance of both sexes in the colour association tasks: developmentally stressed males and females committed more within-trial errors. However, there was no correlation between performance on any of the auditory and colour tasks. Overall, the results suggest that developmental stress can have sex-specific effects on cognition and that aptitude on one cognitive test is not indicative of general cognitive performance.

3.4.1 Auditory learning is impaired by developmental stress in females

Previous developmental manipulations have shown that altering the early auditory and social environment can affect auditory discrimination in both sexes (Njegovan and Weisman 1997; Sturdy et al. 2001). I found that treatment females, despite being raised in the same auditory environment as their control counterparts, took longer to acquire both absolute and relative frequency discrimination tasks. Yet, I did not detect a perceptual impairment as measured by the criterion performance on the absolute frequency task. I cannot state definitively that perception was unaffected as no behavioural design can truly assess physiological perceptual processes (Sturdy et al., 2001). However, I review explanations for the observed pattern of results and discuss potential perceptual and/or cognitive processes that were selectively affected in females.

Although I did not observe a perceptual deficit *per se*, it is still possible that treatment females had impaired perception at the start of testing, but through several thousand additional exposures to the auditory stimuli they refined or improved their perceptual abilities. Unlike zebra finches and black-capped chickadees, starlings are open-ended song learners and are able to modify and learn new songs throughout their
adult lives (Chaiken et al. 1994). Starlings raised in isolation, the same manipulation that induced discrimination deficits in black-capped chickadees and zebra finches (Njegován and Weisman 1997; Sturdy et al. 2001), were able to improve their song to be similar to those of age-matched controls if they were raised in normal social environments for the second year of life (Chaiken and Böhner 2007). Similar behavioural and neural improvements have also been noted in canaries, another open-ended song learner (Leitner and Catchpole 2007). Open-ended song learning may allow starlings to revert to a ‘juvenile pattern of synaptic connectivity’ (Brenowitz and Beecher 2005), which could allow the auditory system and the song-control system of open-ended learners to recover from early-life stress that typically causes permanent deficits in closed-ended learners. There is great diversity in song learning patterns, with several species falling on the continuum between closed to open-ended learning strategies (Brenowitz and Beecher 2005). Nowicki et al. (1998) suggest that song quality in an open-ended species may be reflective of a male’s more recent environmental condition, rather than solely conditions experienced in early development. Thus, the consequences of developmental stress on reproductive fitness could vary drastically between species that are closed- versus open-ended learners.

Developmental stress may also, or alternatively, affect associative learning processes and impair the ability to associate a stimulus with a particular outcome. Association learning in songbirds is often studied using similar colour association tasks as the ones presented here (Boogert et al. 2011; Amy et al. 2012; Isden et al. 2013; Kriengwatana et al. 2015). The fact that treatment females also commit more within-trial errors on these tasks supports the notion that additional learning processes apart from
auditory-specific abilities were affected. Males that experienced the developmental treatment did not exhibit the same pattern. Treatment males’ auditory performance was comparable with control males, but they exhibited more errors on the visual tasks compared to controls. Together, the performance of both sexes across the visual and auditory tasks suggests that males were more robust to the effects of the developmental treatment on auditory development, but association learning was still adversely affected in both sexes. This could be for a few reasons. First, the effects of the developmental treatment may have been less harsh for males and as a result their auditory abilities were spared. However, males and females used in these experiments were equally affected by the developmental treatment (Chapter 2; Farrell et al. 2015b). Birds, of both sexes, in the treatment group had significantly less fat mass during the treatment period and accelerated growth of lean mass (i.e., muscles, organs) after the treatment was terminated compared to control birds (Chapter 2; Farrell et al. 2015b). Therefore, treatment males and females experienced similar physiological changes as a function of the developmental treatment. Yet, there may have been a sex-specific manner in which resources were allocated to, and among, neural processes that my physiological measures would not be sensitive enough to detect.

Due to non-parity in the adult sex ratio, changes in selection pressure may have lead to the development of sex-specific resource allocation strategies. In starlings, the adult population favours males 1.4:1, which supports the notion that females have lower fledgling and over-winter survival in part due to increased competition for resources as the smaller sex (Mayr 1939; Kessel 1957). In wild starling populations, yearling females are much more likely than yearling males to reproduce (Kessel 1957; Flux and Flux
1982), which suggests that only yearling males with superior song will be able to attract females when in competition with older males (Eens et al. 1992). Females raised in stressful environments may shift the allocation of resources away from costly auditory neural development and learning, and instead funnel resources into neuronal development for other cognitive processes and/or somatic growth and maintenance as a strategy to ensure survival until the following breeding season (Schew and Ricklefs 1998; Wingfield et al. 1998). Males may pay the cost to conserve processes necessary for song-learning abilities, as song is crucial for future reproductive success and would win them a greater fitness benefit. But, for a female the benefit received by enhancing her auditory abilities to discriminate the quality of male singers may come at too great a cost if it would compromise her ability to survive to the following breeding season. As the developmental stress hypothesis focuses on males, female brains are comparatively understudied. Future research on the developmental stress hypothesis should include females into measures of brain development to delineate if development of the auditory areas and song-control system is equally affected in both sexes (see Chapter 4; Farrell et al. 2015c).

3.4.2 Sex differences on auditory tasks

Males overall acquired the absolute frequency task faster than females, but females in general outperformed males on the relative frequency task. There could be several non-mutually exclusive explanations for this difference. First, each sex may excel at either task due to sex differences in auditory discrimination. If each sex responded more to particular spectrotemporal features of song, this could translate into learning differences across the auditory tasks. One functional explanation for why sex differences may exist is if the sexes are producing signals which are largely directed at one sex in
particular (Gall et al. 2011). Female starling song is different from male song in several aspects. In general, song sharing is sex-specific in starlings (Hausberger et al. 1995a) and female starlings learn their songs from other females (Poirier et al. 2004). Females do not sing species-specific song themes (Hausberger and Black 1991; Hausberger et al. 1995b) and they tend to direct their song largely towards other females in aggressive contexts (Sandell and Smith 1997). Sex differences in auditory brainstem responses have recently been noted in brown-headed cowbirds, *Molothrus ater*, which could be the result of different selective pressures on each sex (Gall and Lucas 2010; Gall et al. 2011).

Each sex may have favoured certain learning strategies, which affected performance without affecting relative frequency abilities *per se*. Absolute frequency information supersedes relative pitch information in starlings (Hulse et al 1990; MacDougall-Shackleton and Hulse 1996). Males may have tried to solve the relative frequency task by attending only to absolute frequency of the first tone, and therefore their performance is worse due to perseverating by using the previously rewarding strategy dictated by the absolute frequency task. However, if males were more likely to perseverate in general I should have found that males also perseverated more on the colour association task that assessed inhibitory learning. In contrast, there was no sex difference on perseverative errors on the visual tasks. Therefore, if it is perseveration it may be auditory-specific and not a general tendency.

Secondly, females may have applied a samesame rule. In the relative frequency task, one of the unrewarded categories was a ratio of 1.0 (i.e., the same frequency tone played twice). Humans tend to employ a strategy of inhibiting responding to two tones that sound the same when performing frequency ratio discriminations, but birds typically
do not (Weisman et al. 1994). However, females were not employing a same-same rule, because if they were then all exemplars in the ratio 1.0 would have been treated equally. Overall, females inhibited responding more to the unrewarded ratio 1.26, rather than the ratio 1.0. In conclusion, the data support the conclusion that female starlings excel at relative frequency discrimination and this result is not merely a by-product of different learning strategies between the sexes.

Assessing learning performance across multiple contexts allows researchers to determine if developmental stressors target domain-specific or domain-general abilities. There are limitations to the parallels I can draw between cognitive processes used across the learning tasks. To truly compare visual learning to auditory learning, I ideally would have conducted the colour association tasks using an operant conditioning paradigm to hold constant the delivery of reward and punishment. I acknowledge that compared to the auditory tasks, the visual tasks were simpler (i.e., one rule versus learning the reward contingencies for several exemplars), took less time to conduct (i.e., a few weeks versus many months), and were completed after the auditory tasks when the birds were older. The colour tasks also used a more palatable reward (i.e., mealworm) than the operant tasks (i.e., poultry starter). Compared to the auditory tasks, there was minimal punishment for choosing incorrectly on the colour association task. With a larger punishment, treatment birds may have reduced the number of impulsive choice to unrewarded tiles. Nevertheless, this still suggests that the developmental treatment altered learning processes and/or affected impulse control. A recent study in starlings suggest it may be the latter – starlings that showed greater signs of having experienced developmental stress made more impulsive foraging decisions (Bateson et al. 2015).
Neophobia was likely not a mitigating factor in either of the tasks. All birds had extensive training and experience with the both apparatuses prior to testing and neophobia was not affected by early developmental stress in a previous study with starlings (Farrell et al. 2011). All birds were highly motivated to perform the tasks since they were being weight-restricted and birds could readily be seen eating from their food dishes when testing in both auditory and colour tasks were complete for the day. In future, controlling for inherent differences with experimental design will help to delineate these alternative explanations.

3.4.3 Auditory abilities: indicative of a general cognitive ability?

Researchers have postulated that the neural processes that underlie song learning and production could be correlated to other cognitive functions (Catchpole 1996; Nowicki and Searcy 2011). Even if cognitive processes are functionally independent in adulthood, relationships may still arise if neural substrates share overlapping critical sensitive periods in development. A stressful environment and shortage of resources is likely to affect multiple systems and may give rise to developmentally correlated traits (Spencer and MacDougall-Shackleton 2011; Buchanan et al. 2013; Farrell et al. 2015c). Song quality could be a signal of cognitive ability in general (Spencer and MacDougall-Shackleton 2011; Farrell et al. 2015a). To date, there is some support for general cognitive abilities in birds (Keagy et al. 2011a; Isden et al. 2013), but the experiments presented herein and another (Boogert et al. 2011) do not support this notion. Previous studies have also failed to find correlations between song quality and a general index of cognitive performance (Boogert et al. 2011; Keagy et al. 2011a). While I did not examine song quality here, auditory discrimination is a cognitive process that supports song
learning and would affect song quality (Catchpole and Slater 2008). One limitation of previous studies was that the birds were from wild populations whose developmental history, experience, and/or age when completing the tasks was unknown (Boogert et al. 2011). I controlled for these factors and my results are in accordance with previous studies: performance on the auditory discrimination tasks was not indicative of performance on other cognitive tasks. The cognitive processes responsible for birdsong appear to be specific to the domain of song learning.

In order for song learning to evolve it must be a trait with variation, which is heritable, and corresponds to changes in an individual’s fitness. There is some evidence that song learning is heritable (Schmidt et al. 2013b; Woodgate et al. 2014), and that variation in song quality is associated with variation in reproductive success in at least some species (Hasselquist et al. 1996; Reid et al. 2005; Keagy et al. 2011b). Females that are selecting males with high quality song may be mating with males who have genes that encode for better song-control development and song learning (DeVoogd 2004). Interestingly, nest of origin was a random effect that was often significant in most of the statistical models analyzing performance on the auditory tasks. This is consistent with the notion that genetic variation could in part explain variation in auditory learning. In a previous study with the same females used here, it was found that females from the same nest have similar neural responses in auditory forebrain areas to song stimuli (Farrell et al. 2015c). Polygenes could enhance song learning in males, and auditory perception in females. Although non-genetic nest effects could also contribute to this effect (e.g., hormone deposition in eggs, parental care prior to nestling collection, etc.), this result warrants future research.
3.4.4 Conclusions

In conclusion, developmental stress can affect perceptual systems necessary for trait discrimination. Female starlings that experienced unpredictable access to food during development were slower to acquire both absolute and relative frequency discriminations. Males’ auditory abilities as measured here were unaffected by early food-deprivation, unlike females. However, treatment males did exhibit a deficit on the colour association tasks, like their female counterparts, which suggests that developmental stress can cause sex-specific cognitive deficits. A deficit in auditory discriminations in females may explain weaker song preferences and neural response in auditory forebrain areas seen previously in these same female starlings (Farrell et al. 2015c). Developmental stress, by affecting trait discrimination, can have long-term effects on a female’s mating decisions and reproductive success. In future, additional experiments are needed assessing trait discrimination with songs (i.e., recognition of individual motifs, ‘themes’, and individual singers) to confirm that developmental stress also imparts deficits on discriminations with biologically relevant stimuli that contain more than just frequency information.
3.5 References


Chapter 4

4 Developmental stress impairs a female songbird’s behavioural and neural response to a sexually-selected signal

4.1 Introduction

Birdsong is a sexually selected signal that has been researched extensively due to its conspicuous nature. Variation in male song can be explained, in part, by the quality of the environment a male was reared: males reared in stressful environments are unable to devote as many resources to the costly development of the neural structures that enable song learning, ultimately affecting the quality of the song phenotype produced in adulthood (Nowicki et al. 1998; Nowicki et al. 2002). However, the effects of early-life stress on a female’s ability to perceive male song is of equal importance to understanding the evolution of sexually selected traits, and yet is understudied in songbirds (Cotton et al. 2006).

In songbirds, the quality of a male’s song (e.g., repertoire size, song bout length) can reflect other aspects of male condition (Searcy and Andersson 1986). In European starlings (Sturnus vulgaris) male song quality is positively associated with spatial learning, social rank and immune function (Duffy and Ball 2002; Buchanan et al. 2003; Spencer et al. 2004; Farrell et al. 2011). Females that mate with males that possess a high quality song may be receiving direct/indirect benefits by doing so (Searcy 1992a) and therefore a female must be sensitive to variation in song quality. Although the adverse effects of developmental stress on male song are well documented (see Spencer and MacDougall-
Shackleton 2011), the effects of such stress on female song preferences are mixed. Female zebra finches (Taeniopygia guttata) raised in large broods had weaker absolute song preferences (Riebel et al. 2009) and females that experienced a nutritional stressor were less active in mate choice trials (Woodgate et al. 2011), yet compared to their control siblings they preferred songs of equal complexity when sung by unfamiliar singers (Woodgate et al. 2011). In another study female zebra finches raised in large broods preferred the songs of males raised in similar stressful conditions, suggesting an assortative mating strategy based on developmental experience (Holveck and Riebel 2010). Female song sparrows (Melospiza melodia) raised in control conditions were more active when listening to conspecific song than when listening to heterospecific song, but this difference was reduced in females exposed to different developmental stressors (food-restriction or exogenous corticosterone treatment; Schmidt et al. 2013a). Interestingly, there was no difference between stressed and control females in a high-versus low-quality conspecific comparison (Schmidt et al. 2013). With only data from two species, more experimental studies are necessary before general conclusions can be made regarding how developmental stress affects female song preferences and ultimately mate choice decisions.

Male song is adversely affected by developmental stress in part because it hinders neural development of the song-control system (HVC, used as a proper name; robust nucleus of the acropallium, RA; area X; reviewed in Spencer and MacDougall-Shackleton 2011). Female songbirds also have a song-control system, albeit reduced in size (MacDougall-Shackleton and Ball 1999), which may be necessary for the perception of song. Partial lesions to HVC in canaries (Serinus canaria domestica) disrupted song
preferences (Brenowitz 1991; Del Negro et al. 1998) and the size of HVC in canaries and starlings is related positively to the strength of preference towards sexually stimulating songs (Leitner and Catchpole 2002; Ritters and Teague 2003). However, little is known about the effects of developmental stress on the female song-control system. Preliminary work with female song sparrows found that juveniles exposed to early nutritional stress had smaller volume HVC, but this effect was not found in adult females that were subjected to either a nutritional stress or exogenous corticosterone treatment in early-development (MacDonald et al. 2006; Schmidt et al. 2013). These results suggest that HVC may exhibit catch-up growth following an early-life stress, but more work in additional species is needed to substantiate that claim.

In addition to the song-control system, auditory forebrain nuclei (caudomedial mesopallium, CMM; caudomedial nidopallium, NCM) that project to song-control nuclei are necessary for recognizing and processing song (Vates et al. 1996; Bolhuis and Gahr 2006). In zebra finches, lesioning CMM disrupted song preferences, yet lesioning HVC did not (MacDougall-Shackleton et al. 1998). In response to playback stimuli, the neural response within these areas can be measured by assessing expression of the immediate-early gene Zenk (an acronym of zif-268, erg-1, NGFI-A, and Krox-24), which is a transcriptional regulator (Mello et al. 1992). Zenk expression is greatest when birds are listening to their own species song, decreases when listening to heterospecific song, and is minimal in response to tonal stimuli (Mello et al. 1992). Furthermore, levels of Zenk correspond to the sexual potency of an auditory stimulus (Maney et al. 2003) and are therefore a powerful tool to quantify song preferences in addition to behavioural measures. In starlings, behavioural measures of preference show that females prefer to listen to
longer song-bouts over shorter song-bouts and this behavioural preference is in reflected in the Zenk response in NCM – females who heard long song-bouts had more Zenk-immunoreactivity compared to females that heard short song-bouts (Gentner et al. 2001).

More recently, Zenk-immunoreactivity was quantified in females whose early developmental backgrounds were known: song sparrows who were unstressed had significantly more cells expressing Zenk-immunoreactivity in auditory nuclei CMM and NCM when listening to playbacks of conspecific versus heterospecific song in adulthood, yet the amount of Zenk induction was equal across both playback conditions for developmentally stressed females (Schmidt et al. 2013).

Using both behavioural and neural measures of song preference (volumetric analysis of song-control nuclei and Zenk-immunoreactivity within auditory forebrain regions) within the same individual provides a powerful method to observe how early-developmental stress shapes female song preferences. I examined how developmental stress affects female starlings using both these methods. Starlings are an excellent species in which to address these questions for numerous reasons. First, the results from studies of captive and free-living starlings clearly demonstrate that females have a robust preference for male conspecific song, particularly for bouts of song that are long in duration (Mountjoy and Lemon 1991; Mountjoy and Lemon 1996). In addition, song quality is clearly related to reproductive success in this species: males that sing longer song bouts acquire mates faster, their female partners lay clutches sooner, and they have larger clutches (Eens et al. 1991; Mountjoy and Lemon 1996). Females are sensitive to variation in male song quality, as measured in choice-tests (Gentner and Hulse 2000), and through the amount of Zenk induction to playbacks of conspecific songs of long and short
duration (Gentner et al. 2001; Sockman et al. 2002). And last, experiencing stressful food-restriction in early development has known effects on song behaviour and other cognitive abilities in starlings (Buchanan et al. 2003; Spencer et al. 2004; Farrell et al. 2011).

In my study, I manipulated early-developmental conditions through an unpredictable food supply paradigm. This unpredictable food supply paradigm is known to have long-term effects on the songs of male European starlings and song sparrows, and induces changes in growth rates, humoral immune response, metabolic rates, and reproductive physiology (Buchanan et al. 2003; Farrell et al. 2011; Schmidt et al. 2012; Schmidt et al. 2014; Farrell et al. 2015b). After the developmental treatment ceased, I measured females’ preferences for songs several months later using an operant-conditioning paradigm in two comparisons: (1) conspecific versus heterospecific song (i.e., canary song) and (2) short song-bouts versus long song-bouts of conspecific songs. After behavioural testing was complete, I then played either conspecific or heterospecific songs to birds prior to quantifying the Zenk response within the auditory forebrain regions of CMM and NCM. I also measured the volumes of three song-control nuclei (HVC, RA, area X) in relation to overall telencephalon volume and compared HVC size to the strength of preferences as measured in the operant-conditioning tasks. I hypothesized that females raised in stressful early-life environments would exhibit weaker behavioural and neural responses towards sexually potent signals compared to their control counterparts. Moreover, these weaker responses could be correlated with song-control system volumes and I hypothesized that females with larger HVC volume
will, regardless of early-life conditions, exhibit the strongest preferences for sexually relevant songs.

4.2 Methods

4.2.1 Subjects

Subjects are the same females birds as those described in Chapters 2 and 3. One female raised in the treatment group died before completing the preference experiment (Table 4-1). In the center of the colony room, I individually housed 9 adult starlings (5 male, 4 female) to serve as song tutors for the duration of the experiment. Therefore, all females had identical song exposure prior to any preference testing.

<table>
<thead>
<tr>
<th>Developmental treatment</th>
<th>Experienced food-restriction as a result of cognitive testing (i.e., cognitive treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
</tr>
<tr>
<td>Treatment</td>
<td>6</td>
</tr>
</tbody>
</table>

4.2.2 General procedural timeline

Birds were kept on a long day photoperiod after the treatment was over until approximately 10-12 months of age. Birds were then were switched to a short day photoperiod (LD 10:14) for a minimum of 21 weeks in order to dissipate photorefractoriness (Dawson and Goldsmith 1983). To photostimulate birds, I then switched them to a long day photoperiod (LD 15.5:8.5) for a minimum of two weeks prior to their first preference test. After song preference testing was complete (about 2-4 days later), birds were moved to an acoustic isolation chamber. After 48 h in isolation,
the Zenk playback study was conducted and the brain was collected for further histology and immunohistochemistry analysis (see below for detailed procedures). All experimental data were therefore collected within approximately 5 weeks of exposure to long days. I confirmed that all birds were in reproductive condition during the song preference testing and Zenk playback procedures by visually examining their ovaries after birds were euthanized. All birds had enlarged ovaries with a visible follicular hierarchy.

Between the ages of approximately 6-12 months, a subset of birds (Table 4-1) in this study participated in a separate study assessing auditory discrimination abilities (Chapter 3; Farrell et al. 2015a). In brief, auditory abilities were assessed in an operant-conditioning paradigm using a different apparatus than the one described here. Birds completed tasks assessing abilities to discriminate absolute frequency and relative frequency of synthetic pure tones (see Sturdy et al. 2001). Birds were tested daily, and therefore were food-restricted to 85-90% of their *ad libitum* body weight to ensure high motivation to complete testing. This extended period of food-restriction was an unintended additional developmental stressor and I have included whether or not birds experienced food restriction as a result of these cognitive tests as a fixed effect in all analyses. Hereafter, I will use the terms “treatment” and “cognitive treatment” to delineate any effects caused by the developmental treatment or as a result of the cognitive testing, respectively.

### 4.2.3 Experiment 1: behavioural song preferences

I modeled the behavioural study after a technique described by Gentner et al. (2000) in which birds select the songs they listen to via an operant-conditioning apparatus. This technique provides strong evidence for preference as the bird is making
an active choice to listen to the song, rather than monitoring behaviour while a song is passively played to her. Here, the female is allowed to sample songs in a parallel, rather than a serial fashion, which is more representative of wild mate searching strategies (Dale et al. 1990) I conducted a (1) conspecific versus heterospecific song comparison and (2) a conspecific long song-bout versus short song-bout comparison. Each subject completed both song preferences assays in an order that was randomized across treatment groups. After completing the first assay, birds were returned to their colony room for approximately 1 week at which point they were returned to complete the second assay.

4.2.3.1 Song stimuli

All starling songs were from recordings of one male starling from outside the population where the subjects were sampled (Figure 4-1; detailed recording procedures

Figure 4-1 Spectrogram of ‘starling short song 1’ that was used in both the behavioural and neural response to conspecific song playbacks.
outlined in Gentner & Hulse, 1998). Briefly, I used 10 song bouts that have been previously used in other studies assessing adult female starlings’ preference for variation in male song (Gentner & Hulse, 2000; Gentner, Hulse, Duffy, & Ball, 2001). Half of these song bouts are defined as long bouts, which is to say they are longer in duration than the other half of the bouts, which were short bouts (Table 4-2). Adult female starlings have been found to prefer to listen to the long bouts more than the short bouts in an operant-choice assay and show increased Zenk-immunoreactivity in the ventral part of auditory forebrain area NCM (NCMv) when listening to long versus short song bouts (Gentner and Hulse 2000; Gentner et al. 2001).

Table 4-2 Song stimuli used in both the behavioural and Zenk playback experiments

<table>
<thead>
<tr>
<th>Song stimulus</th>
<th>Mean song duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canary 1</td>
<td>26.1</td>
</tr>
<tr>
<td>Canary 2</td>
<td>12.0</td>
</tr>
<tr>
<td>Canary 3</td>
<td>17.1</td>
</tr>
<tr>
<td>Canary 4</td>
<td>19.7</td>
</tr>
<tr>
<td>Canary 5</td>
<td>10.0</td>
</tr>
<tr>
<td>Starling Short 1*</td>
<td>26.9</td>
</tr>
<tr>
<td>Starling Short 2</td>
<td>29.9</td>
</tr>
<tr>
<td>Starling Short 3*</td>
<td>31.4</td>
</tr>
<tr>
<td>Starling Short 4</td>
<td>21.0</td>
</tr>
<tr>
<td>Starling Short 5</td>
<td>14.2</td>
</tr>
<tr>
<td>Starling Long 1*</td>
<td>47.0</td>
</tr>
<tr>
<td>Starling Long 2</td>
<td>77.0</td>
</tr>
<tr>
<td>Starling Long 3*</td>
<td>60.5</td>
</tr>
<tr>
<td>Starling Long 4*</td>
<td>68.4</td>
</tr>
<tr>
<td>Starling Long 5</td>
<td>72.3</td>
</tr>
<tr>
<td><strong>Average Canary</strong></td>
<td><strong>17.0 (4.5)</strong></td>
</tr>
<tr>
<td><strong>Average Short</strong></td>
<td><strong>24.7 (8.1)</strong></td>
</tr>
<tr>
<td><strong>Average Long</strong></td>
<td><strong>65.0 (7.0)</strong></td>
</tr>
</tbody>
</table>

Mean song duration (s) is listed for each stimulus and the bottom three rows are the average mean song duration (SD) for a stimulus song in that category.

*Denotes songs that were selected for the starling playback used in the Zenk experiment.
I chose to use canary song in the heterospecific song preference comparison and Zenk-immunoreactivity study. Although canaries sing shorter songs on average than starlings, their song duration is within the range of starling song (Table 4-2; Figure 4-2) and they sing across similar frequency bandwidths. Yet, their song is easily discriminable from starling song and I therefore predict my females should clearly prefer the starling song in the comparisons. All canary songs in the study were sampled from commercially available recordings.

Figure 4-2 Spectrogram of ‘canary song 1’ that was used in both the behavioural and neural response to heterospecific song playbacks.

Both starling and canary stimuli were processed using Audacity software (version 2.0.2.; D Mazzoni; http://audacity.sourceforge.net). Stimuli were high-pass filtered to remove background noise (200 Hz cutoff). The leading and trailing 5 ms of the songs
were tapered to remove transients and peak amplitude was equalized. Stimuli were sampled at 44.1 kHz with 16-bit resolution and made into .wav audio files.

4.2.3.2 Preference testing apparatus

The apparatus used in this study can be seen in Figure 4-3. During behavioural testing, birds were held in a wire cage (76 cm X 46 cm X 45 cm) in a separate room. On the front of the cage (the only side without a nestbox) I placed a food dish and water bottle at all times. On the other three sides of the cage, I placed 3 nestboxes flush against the test cage. Each nestbox (18 x 23 x 30 cm) was affixed with a motion-activated perch mounted at a right angle to the front of the box. I made small openings to allow for each

![Figure 4-3 A schematic of apparatus used in the behavioural song preference experiments. Three nestboxes were equipped with perches that were sensitive to motion via infrared-sensors. When a bird landed on a perch, it would register a response with a computer controlling song stimuli that would then play from a speaker inside the corresponding nestbox. More detailed information about the apparatus is in the methods section.](image)

motion-activated perch to protrude inside the bird’s cage such that they could use it to perch on. Inside each of the nestboxes used during the playbacks was a wireless speaker (Mini Bullets II, Grace Digital Audio, CA, USA); the silent box did not contain a speaker.

The perches affixed to the nestboxes were the only available perches inside the cage. The perches were constructed of a 1.25 cm diameter plastic dowel 20 cm in length and were mounted 10 cm below the nestbox entrance holes. An LED infrared emitter (Honeywell SEP8736) and receiving transistor (Honeywell SDP8436) were at opposite ends of the perch, housed in small pieces of pipe protruding vertically from the main perch. Each LED-transistor circuit was connected to a Propeller Proto Board (Parallax Inc., Rockland, CA), which would detect when the LED beam-transistor connection was broken and record the duration of this interruption. These data were then delivered via a serial connection to a PC computer. A custom computer script (Python; PySerial and PyGame libraries used) recorded which of the three beams was interrupted, the duration of the interruption, and played a song from the corresponding category of songs assigned to that perch for the session (no song in the case of the silent box).

4.2.3.3 Procedure

Females were placed into the test cage in the morning and were allowed to spend their time sitting on any of the three perches, sitting on the floor, or eating/drinking at the front of the cage. The preference tests were each divided into two blocks. Each block consisted of whichever came first: 30-minutes of cumulative playbacks (time spent on silent perch did not count) or 1-day in the test chamber. In the first block, two of the boxes were randomly assigned to play one of the categories of songs and the third
remaining box was a silent control. Songs would only play from a nestbox if the bird landed on the corresponding perch for at least 1.5 s (this delay was to prevent transient stops from triggering playbacks) and would stop playing once the bird left the perch. During the second block, the song contingencies were switched. For example, the box that played conspecific songs in block 1 would now switch and play heterospecific songs, and vice versa for the other box. The silent control remained the same across both blocks. I switched the song-box contingencies between blocks 1 and 2 in order to control for any location biases. Therefore, if a bird truly preferred to listen to a particular category of songs then their preference should be apparent in both blocks. In addition, I randomized the six possible nestbox-song category combinations across subjects during the first block of testing to control for any location preferences. While the silent perch remained the same within a preference test, I never assigned the same perch to be the silent control for the second preference test.

4.2.3.4 Response measures

During testing, the computer would record each perch event and log which perch was active, the song stimulus played, and the duration of the playback. I recorded time spent on the two active perches that led to song playback, and time not spent on these perches (either on the silent perch or elsewhere in the cage). The response measures included the total amount of time spent on each playback perch and total number of visits to each perch. I calculated the strength and directionality of preference each female showed towards conspecific song (over heterospecific song) or long song-bouts (over short song-bouts) by determining the proportion of time spent listening to each target category song over total time spent listening to all song types, and proportion of visits to
the perch associated with the category song over total number of visits to both playback perches. A score over 0.50 would indicate no clear preference for either conspecific song (over heterospecific song) or long song-bouts (over short song-bouts).

4.2.4 **Experiment 2: neural activation in relation to song**

To corroborate the behavioural preference results I measured the induction of Zenk-immunoreactivity in females when listening to conspecific or heterospecific songs (i.e., canary song). I assigned subjects to one of the playback conditions counterbalancing across developmental treatment, if they participated in the cognitive treatments, and the order in which they performed the behavioural preference comparisons.

4.2.4.1 **Playback procedure**

Prior to the playback session, I housed birds individually for 48 h in a cage within a sound attenuation chamber with access to food and water *ad libitum* (Eckel Noise Control Technologies, Morrisburg, ON, Canada, model AB-2000 S). Lights in the chamber were synchronized to the same photoperiod as the colony room and two speakers (Spin-45 speakers, Labtec, WA, USA) were placed on either side of the cage. On the floor of the chamber was a microphone that allowed us to monitor the playback session. This was to ensure the playback was successful and that the bird did not vocalize during the session. There was minimal to no vocalizations from all subjects and therefore I do not account for vocal output as a factor in the analyses.

To create the playback stimuli for the Zenk playbacks, I used the same starling and canary songs used in the conspecific versus heterospecific behavioural preference comparison (Table 4-2). The starling playback consisted of 5 starling songs, each separated by 4.6 s of silence, repeated 7 times. The canary playback consisted of the 5
canary songs, each separated by 3 s of silence, repeated 18 times. Therefore, the starling and canary playbacks had approximately equal sound:silence ratios of 1639:161 s and 1698:102 s, respectively.

All playback sessions commenced at 13:30 h daily. Fifteen minutes prior to playback, I turned the chamber lights off (to deter subjects from vocalizing) and began monitoring the auditory environment within the chamber. I played either a starling or canary stimulus for 30 minutes, followed by 30 minutes of silence to maximize Zenk induction (Mello and Clayton 1994). All sounds files were played at a maximum of 70 dB SPL as measured from the bird’s position in the closed chamber.

Once the playback session was completed, birds were deeply anesthetized with isoflurane and transcardially perfused with phosphate buffered saline (PBS; pH 7.4) followed by 4% buffered paraformaldehyde. Brains were then removed from the skull, stored in 4% paraformaldehyde for 24 h and then in 30% sucrose (in PBS) for 48 h. Brains were then frozen on powdered dry ice and then stored in at -80 °C until later analysis. Prior to histological and immunohistochemistry analyses, each brain was cut in half along the sagittal plane and either the left or right hemisphere was selected randomly for further analyses.

4.2.4.2 Zenk immunohistochemistry

Using a cryostat, I sectioned brains in the sagittal plane in 40 µm sections. I put alternating sections into 0.1 M PBS for Zenk immunohistochemistry and Nissl histology (explained below). I ran immunohistochemistry in 6 runs, counterbalanced across conditions. First, free-floating sections were washed twice in 0.1 M PBS, followed by a 15 min incubation in 5% H₂O₂. I then washed the tissue three times in 0.1 M PBS prior to
a 1 h incubation in 10% normal goat serum (Vector, Burlingame, CA, USA) diluted in 0.3% Triton in PBS (PBST). Sections were then incubated for 20 h in the primary antibody (anti-Egr-1 sc-189, Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:4000 in 0.3% PBST. I then washed section three times in 0.1% PBST and followed this with a 1 h incubation in the biotinylated secondary antibody diluted 1:250 in 0.3% PBST (goat anti-rabbit IgG, Vector). Following three washes in 0.1% PBST, sections were incubated in avidin-biotin horseradish-peroxidase complex (Vector Elite ABC kit) for 1-hour and then washed twice in 0.1% PBST. Section were then visualized with diaminobenzidine solution (SigmaFast DAB) and washed 5 times in 0.1 M PBS and later mounted onto gelatin-coated microscope slides. Last, mounted sections were serially dehydrated with graded ethanols, cleared in a solvent (Harleco Neoclear, EMD Chemicals), and protected by affixing a coverslip with Permount (Fisher Scientific).

4.2.4.3 Zenk quantification

Using a similar protocol as previous studies (Gentner et al. 2001; Maney et al. 2003; Hernandez and Macdougall-Shackleton 2004; McKenzie et al. 2006; Schmidt et al. 2013), I measured the number of Zenk-immunoreactive cells within three regions of the telencephalon that have been found to show increased levels of immunoreactivity in response to conspecific song (Mello and Clayton 1994; Gentner et al. 2001; Maney et al. 2003): the CMM, dorsal NCM (NCMd) and ventral NCM (NCMv). In female starlings, NCMv has been shown specifically to respond to variation in quality of male conspecific song (Gentner et al. 2001).

I began by measuring the most medial section by which NCM was visibly attached to the rest of the brain and field L2 was visible due to its lack of
immunoreactivity (Mello and Clayton 1994). In this section, and then next 5 lateral to it, I sampled each region by focusing on the center of the structure. Therefore, I measured six sections (alternating every 40 µm) for a total sampling area that spanned 480 µm wide mediolaterally (a comparable distance covered in other studies; Maney et al. 2003; Hernandez and Macdougall-Shackleton 2004; Schmidt et al. 2013). An observer blind to treatment and playback condition took images (0.515 x 0.386 mm) within each sampling region using a Leica Digital CCD camera mounted on a Leica DM5000B light microscope using a X20 objective lens.

Using ImageJ software (National Institute of Health, USA), I counted the number of Zenk-immunoreactive cells within each image. First, I converted images to 8-bit grayscale and then counted the number of particles with an optical density that was above a threshold value using the threshold feature in ImageJ. Due to variability in the intensity of background staining, this threshold was set manually for each image such that a blind observer agreed that the highlighted pixels matched where nuclei were visible (Schmidt et al. 2013). To set the exclusion limits for cell size, I calculated the size of 60 cells (three cells per bird from randomly chosen sections) and then set the maximum/minimum cell size as the average plus/minus two standard deviations. Exclusion limits for sphericity were set at 0.65.

4.2.4.4 Nissl histology and quantification

I mounted the alternate series of brain sections onto gelatin-coated microscope slides and let them air dry. Next, sections were stained with thionin, serially dehydrated in graded ethanols, cleared in a solvent (Harleco Neoclear) and protected by affixing a coverslip with Permount (Fisher Scientific). I quantified the volume of the three song-
control nuclei of interest: HVC, area X, and the robust nucleus of the archistriatum (RA). Volumes were calculated by taking images from every section that contained the nuclei of interest using a 5-megapixel digital camera (Spot Idea; Diagnostics Instruments) connected to a Zeiss Axiophot microscope. Using ImageJ, I traced the outlines of the nuclei and then using the formula for the volume of a frustum I estimated the total volume of the nuclei. As every second section was measured, volume estimates were made using areas spaced at 80 µm intervals. To estimate total brain size (telencephalon volume), microscope slides were scanned at 1200 dpi on a flatbed scanner with a transparency adapter. Using ImageJ, I traced the telencephalon in every 20th section (800 µm sampling interval), and estimated total telencephalon volume using the formula for the volume of a frustum.

4.2.5 Data and statistical analysis

All data were analyzed using linear mixed models. I examined the effects of developmental treatment and if birds participated in the cognitive experiments (i.e., cognitive treatment). Therefore, developmental treatment, cognitive treatment, and their interaction were entered as fixed effects in all models. Models were subsequently tailored to each experiment by adding additional fixed effects of interest (and any of their higher order interactions with developmental treatment and cognitive treatment) and covariates of interest.

For the behavioural preference tests, I ran separate analyses for each preference test on each dependent variable. Block was entered as a repeated factor (unstructured covariance structure) and as a fixed effect. Nest of origin, order of the preference test, and the location of the silent perch were initially entered as random effects, but none of these
variables contributed significantly to any of the models so they were removed from the analysis. In addition to comparisons across the treatments, I ran one-sample t-tests for each treatment to determine if the preference scores differed significantly from chance (i.e., a score of 0.50). To determine if birds across treatments groups were equally motivated during the test I ran a full model using the total amount of song triggered during the playbacks as the dependent variable.

Zenk-immunoreactivity was analyzed using the total number of Zenk-immunoreactive cells in each region as the dependent variable. Fixed effects of brain region (CMM, NCMd, NCMv), playback (starling/canary), and the covariate of telencephalon volume were entered along with random effects of nest of origin and hemisphere (left or right) analyzed. Hemisphere did not contribute significantly and was removed from the analysis.

I calculated for each song-control nucleus the volume of the nucleus as a proportion of overall telencephalon size as the dependent variable. I included region as a fixed effect and included it with all higher-order interactions with developmental treatment and if birds participated in the cognitive treatment. Random effects of nest of origin and hemisphere analyzed were first included, although neither of these effects contributed significantly to the model and were removed from the analysis. Last, I ran HVC size (as a proportion of telencephalon size) as a predictor against the preference ratios for both the conspecific/heterospecific and long/short song bout comparisons, while controlling for nest of origin. Nest remained in the conspecific/heterospecific analysis but was removed from the long/short analysis.
All models were first constructed as fully loaded models (all predictors and interactions included). I then calculated the minimal adequate model by removing non-significant predictors using log-likelihood ratio tests to maintain parsimony and improve model fit (Chapter 2; West et al. 2007). All significant results reported here are the minimal adequate models with restricted maximum likelihood estimation. Statistics reported for non-significant effects are the values from the full model prior to removal. Post-hoc tests were carried out using Bonferroni corrections. I checked residuals for each model against a normal distribution and found no violations of normality. SPSS Statistics (v. 21) was used for all analyses.

4.3 Results

4.3.1 Behavioural song preferences: starling vs. canary song

Developmental treatment did affect preference for conspecific song: control females spent proportionally more time listening to and visiting the perch associated with starling songs than treatment females (Time: $F_{1,17} = 6.501, P = 0.021$; Figure 4-4a; Visits: $F_{1,17} = 4.899, P = 0.041$). Moreover, control females listened to starling song significantly more than chance levels ($t_7 = 2.498, P = 0.041$) while treatment females did not ($t_{11} = -0.473, P = 0.646$). In addition to the effects of early life treatment, females that did not participate in the cognitive treatment spent proportionally more time listening to and visiting the perch associated with starling songs than females that did (Time: $F_{1,17} = 4.808, P = 0.043$; Visits: $F_{1,17} = 4.694, P = 0.045$). There was no interaction between developmental treatment and cognitive treatment for either measure of time listening or visits made to each perch.
Figure 4-4 Preference ratios for the (a) conspecific versus heterospecific (i.e., canary) song comparison and, (b) the long versus short starling song bout comparison. Preference ratios were calculated by taking the total duration spent listening to (a) starling song or, (b) long song bouts, over the total duration of all song played. Raw data points for individuals are indicated by the open circle (control) and filled triangles (treatment). A preference ratio above 0.50 indicated a preference for (a) starling song or, (b) long song bouts. Bars represent group means with error bars represented as ± SE. * P < 0.05; An asterisk directly above the bar denotes that the mean response for that treatment group was significantly different from a preference ratio of 0.50.
4.3.2 Behavioural song preferences: high vs. low quality starling song

In the within starling song preference test, neither developmental treatment (Time: $F_{1,15} = 1.370, P = 0.260$; Figure 4-4b; Visits: $F_{1,15} = 1.174, P = 0.296$) or cognitive treatment (Time: $F_{1,15} = 0.026, P = 0.874$; Visits: $F_{1,15} = 1.151, P = 0.300$) were significant as main effects. However, there was a significant interaction between developmental treatment group and cognitive treatment with regard to both time spent listening to high quality song ($F_{1,15} = 18.897, P < 0.001$) and visits made to the high quality perch ($F_{1,15} = 23.748, P < 0.001$). The nature of the interaction is difficult to interpret, as the group I predicted to have the least exposure to stressors (control females who did not participate in the cognitive experiments) and the group with the most exposure to stressors (treatment females that did participated in the cognitive experiments) appear to have both preferred the perch that played less complex starling song. Due to the small sample sizes when considering both these factors, it appears that some females showed a strong preference for one of the starling song types, but the direction of the preference is not consistent at the level of developmental treatment or cognitive treatment. Lastly, birds were equally active during both preference tests regardless of status of developmental treatment or cognitive treatment (all $P$-values $> 0.09$).

All the statistics above were conducted using the preference ratio scores. For information regarding descriptive statistics describing the average amount of time spent on each perch, number of perch visits, and visit duration organized by developmental treatment and participation in the cognitive treatments, refer to Tables 4-3 and 4-4, respectively.
**Table 4-3** Mean (± SE) time on perch, number of visits, and visit duration to each song stimulus (starling-canary comparison; long-short song bout comparison) by treatment condition

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Starling (s)</th>
<th>Canary (s)</th>
<th>Starling (s)</th>
<th>Canary (s)</th>
<th>Starling (s)</th>
<th>Canary (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>684.76 (158.93)</td>
<td>342.47 (131.00)</td>
<td>50.13 (10.37)</td>
<td>44.38 (15.94)</td>
<td>15.65 (2.35)</td>
<td>8.06 (0.91)</td>
</tr>
<tr>
<td>Treatment</td>
<td>617.51 (129.76)</td>
<td>583.24 (107.13)</td>
<td>46.63 (8.47)</td>
<td>68.21 (13.02)</td>
<td>13.36 (2.00)</td>
<td>8.92 (0.74)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Long (s)</th>
<th>Short (s)</th>
<th>Long (s)</th>
<th>Short (s)</th>
<th>Long (s)</th>
<th>Short (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>527.55 (159.92)</td>
<td>602.57 (144.85)</td>
<td>42.13 (11.58)</td>
<td>52.69 (16.47)</td>
<td>13.88 (2.36)</td>
<td>12.44 (1.29)</td>
</tr>
<tr>
<td>Treatment</td>
<td>560.42 (130.57)</td>
<td>515.20 (118.27)</td>
<td>37.96 (9.45)</td>
<td>55.21 (13.44)</td>
<td>14.99 (2.05)</td>
<td>11.20 (1.10)</td>
</tr>
</tbody>
</table>

All data are presented in raw form. Statistical analyses reported in the main text were conducted on ratio scores that were calculated for each individual based on their raw data.

**Table 4-4** Mean (± SE) time on perch, number of visits, and visit duration to each song stimulus (starling-canary comparison; long-short song bout comparison) by participation in the cognitive experiments from Chapter 3

<table>
<thead>
<tr>
<th>Cognitive Treatment</th>
<th>Starling (s)</th>
<th>Canary (s)</th>
<th>Starling (s)</th>
<th>Canary (s)</th>
<th>Starling (s)</th>
<th>Canary (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>554.87 (133.99)</td>
<td>560.51 (113.44)</td>
<td>50.27 (8.84)</td>
<td>67.86 (13.37)</td>
<td>11.57 (2.01)</td>
<td>8.31 (0.75)</td>
</tr>
<tr>
<td>No</td>
<td>753.85 (148.13)</td>
<td>397.00 (125.41)</td>
<td>45.28 (9.77)</td>
<td>47.44 (15.10)</td>
<td>17.39 (2.11)</td>
<td>8.96 (0.89)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cognitive Treatment</th>
<th>Long (s)</th>
<th>Short (s)</th>
<th>Long (s)</th>
<th>Short (s)</th>
<th>Long (s)</th>
<th>Short (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>560.71 (136.38)</td>
<td>628.95 (122.412)</td>
<td>43.77 (9.83)</td>
<td>63.14 (13.88)</td>
<td>12.75 (2.00)</td>
<td>11.56 (1.11)</td>
</tr>
<tr>
<td>No</td>
<td>530.84 (150.78)</td>
<td>453.84 (135.33)</td>
<td>34.56 (10.87)</td>
<td>43.28 (15.34)</td>
<td>16.87 (2.30)</td>
<td>11.94 (1.30)</td>
</tr>
</tbody>
</table>

All data are presented in raw form. Statistical analyses reported in the main text were conducted on ratio scores that were calculated for each individual based on their raw data.
4.3.3 Zenk activation

Overall, females that listened to starling songs had significantly more Zenk-immunoreactive cells than those that heard canary song (Playback: $F_{1,46.06} = 71.768, P < 0.0001$; Figure 4-5a), and this was evident in every area measured (Area x Playback: $F_{2,38.57} = 9.128, P < 0.001$; Figure 3a). While there was no difference overall between developmental treatment groups ($F_{1,46.02} = 0.784, P = 0.380$), there was a significant interaction between playback and treatment ($F_{1,29.41} = 4.270, P = 0.048$; Figure 4-5b). Control females that were played starling song had significantly more Zenk-immunoreactive cells than treatment females who also heard starling song ($P = 0.028$); there was no difference in activation between the treatment groups when listening to canary song ($P = 0.175$). Birds that participated in the cognitive treatment had reduced Zenk activation ($F_{1,47.46} = 16.883, P < 0.001$) and there was a significant developmental treatment by cognitive treatment interaction ($F_{1,43.18} = 8.465, P = 0.006$). Control females that participated in the cognitive treatment had less activation than those controls females who did not ($P < 0.0001$); however, there was no difference between treatment females ($P = 0.722$). I found that there was a difference in activation across the areas measured ($F_{2,38.57} = 41.129, P < 0.0001$): CMM had significantly more activation than both NCMd ($P < 0.001$) and NCMv ($P < 0.0001$), and NCMd had more activation than NCMv ($P < 0.0001$). Moreover, control females were found to have more activation in NCMv than treatment females regardless of which playback they heard (Treatment x Area: $F_{2,38.57} = 3.671, P = 0.035$; post-hoc for NCMv: $P = 0.024$). Lastly, nest of origin was significantly related to the amount of Zenk-immunoreactive cells expressed (Wald $Z = 1.991, P = 0.046$).
Figure 4-5 Zenk-immunoreactivity as measured across the starlings and canary playback conditions (a) by auditory forebrain regions and (b) by developmental treatment (Control: gray bars; Treatment: white bars). Zenk was measures across the caudomedial mesopallium (CMM; dark gray bars), and the ventral and dorsal caudomedial nidopallium (NCMv and NCMD, light gray and white bars, respectively). Bars represent means ± SE. * P < 0.05, ** P < 0.01.
4.3.4 Song-control system nuclei volumes

For song-control region volumes I found no significant effect of developmental treatment ($F_{1,59} = 0.515, P = 0.476$; Figure 4-6a), cognitive treatment ($F_{1,59} = 3.238, P = 0.077$; Figure 4-6b), or any of their higher-order interactions with region.

Figure 4-6 The effects of (a) developmental treatment (Control: gray bars; Treatment: white bars) and (b) participation in the cognitive treatments (No: gray hatched bars; Yes: white hatched bar) on the volume of the nuclei of the song-control system. Volumes were measured for HVC, area X, and robust nucleus of the arcopallium (RA) and are presented here corrected for telencephalon size. Bars represent means with ± SE.
(all $P$’s > 0.580). However, there were significant differences in size between the three song-control nuclei measured (Region: $F_{2,59} = 195.853, P < 0.0001$). Telencephalon size did not differ between treatments ($F_{1,16} = 2.014, P = 0.175$), if birds participated in the cognitive treatment ($F_{1,16} = 0.032, P = 0.859$), or their interaction ($F_{1,16} = 0.028, P = 0.869$).

4.3.5 HVC and song preferences

The size of HVC was positively related to the preference ratio in the conspecific/heterospecific comparison ($F_{1,16.28} = 6.622, P = 0.020$; Figure 4-7a). Females with a larger HVC had a stronger preference to listen to starling song over canary song. However, HVC size was not related to the preference ratio in the short/long starling song comparison ($F_{1,17} = 1.202, P = 0.288$; Figure 4-7b).

4.4 Discussion

A stressful environment experienced during the early months of life, and even in the later juvenile stage (i.e., cognitive treatment), affected female starlings’ behaviour and neural response to male conspecific song. Females that experienced stress at any point in development had reduced preferences to their own species song over canary song compared to control females in the behavioural assay. Moreover, these behavioural results were paralleled in the neural assay using Zenk-immunoreactivity: both forms of stress reduced the number of Zenk-immunoreactive cells overall in the auditory telencephalon when listening to conspecific song. While stress did not cause any reduction in volume of the song-control nuclei or overall telencephalon size, HVC size
Figure 4-7 The relationship between HVC volumes (as % of overall telencephalon volume) and the behavioural preference ratio calculated for the (a) starling versus canary song comparison, and (b) short versus long starling song bout comparison was positively related to the strength of the preference for listening to one’s own species song. However, there was no consistent effect of stress on preference for listening to long
song-bouts over short song bouts, nor any relationship between HVC size and the strength of said preference.

### 4.4.1 Behavioural preferences

My study provides evidence that developmental stress can alter the perception of biologically relevant signals as measured through an active-choice assay. Operant conditioning, the technique used here, is a powerful way to assess song preferences and has been known to reliably reflect song preferences in several species (zebra finches: Riebel and Slater 1998; starlings: Gentner and Hulse 2000; chaffinches, *Fringilla coelebs*: Riebel 2000). Various methods have been used to study song preferences. Most studies passively playback song stimuli to a female and quantify the number of copulation solicitation displays she performs, phonotaxis, or measures of general activity levels (Searcy 1992b; Byers and Kroodsma 2009). While these are valid methods for studying song preferences, the operant conditioning method can assess if cognitive aspects of preference are affected. Song preferences are likely to have a cognitive component and can be difficult to observe using a passive playback design. In the wild, females are known to actively engage in different sampling strategies to sample prospective males (Gibson and Langen 1996; Cotton et al. 2006). For example, female pied-flycatchers (*Ficedula hypoleuca*) sample multiple males and revisit males for several days prior to making a mate-choice decision (Dale et al. 1990). While monitoring behaviour in a more passive playback design is also informative, a female is only able to make a binary choice – she can respond to the stimulus or not (Gentner and Hulse 2000). Using a threshold rule a female may respond to any stimulus that meets a certain performance criterion (Gibson and Langen 1996) and therefore by responding to one stimulus
it does not rule out that she may respond to others. In the behavioural preference assay, females had to learn to associate the spatial location of a perch with a particular song stimulus, which is comparable to a wild-caught female starling associating the song repertoire of a male with a particular breeding location.

While developmental stress reduced a female’s preference towards conspecific song over heterospecific song, there was no difference in preference towards conspecific long versus short song bouts. Some females clearly preferred one song type to the other, although the directionality of the preference was not related to the developmental treatment or cognitive treatment. While adult female starlings are known to prefer long song bouts over short song bouts (Gentner and Hulse 2000), I was unable to replicate this finding in my hand-reared birds despite using the same stimulus songs from the original study by Gentner and Hulse (2000). All of the birds had the same exposure to adult starling song during development, yet it is worth noting that the rearing conditions are still atypical from those of a free-living starling. Social experience has a large influence over song tutor choice and song acquisition in starlings. Adult influence over the development of young starlings’ song is increased when direct social contact is available (i.e., housed in the same cage) and decreases proportionally to the number of young present per adult (Bertin et al. 2007; Bertin et al. 2009). In my study, there were approximately 5 young for every adult tutor (including male siblings that were not used in these experiments here) and all birds were individually housed during the experiments such that adults and young were never housed together. It is likely that my female starlings were strongly influenced by the songs of same-aged conspecifics compared to the adult tutors present (Poirier et al. 2004). Starlings raised without adult influence tend
to sing songs with atypical features and their songs are categorized by conspecifics differently when being compared to wild birds’ songs (Chaiken et al. 1997). Therefore, the starling songs used in these experiments (recorded from an adult male outside the population) were perhaps qualitatively different from the songs my birds were largely exposed to in their rearing environment. Birds may have preferred elements of one song-type that was similar to songs heard in their rearing environment and this may explain the strong preferences exhibited by some females. Considering the similarity of stimulus songs to songs heard in the early-life environments is an important consideration for all future studies. In zebra finches, the data provide weak support for the hypothesis that developmental stress could affect song preferences. However, female zebra finch song preferences are strongly shaped by the songs they hear in development (Lauay et al. 2004). Typically, the similarity of stimulus songs to tutors songs heard in development is not controlled for and this could in part explain the assortative mate preference observed of females raised in low-quality environments prefer the songs of males raised in similar environments to themselves (Holveck and Riebel 2010).

4.4.2 Zenk activation

The Zenk experiments were conducted over 1 year after the developmental treatment was terminated and a minimum of 5 months after the cognitive treatments ended (Chapter 3; Farrell et al. 2015a). While it had been several months to almost a year after the stressors were removed, the birds were still experiencing long-term adverse effects. Overall, the levels of Zenk-immunoreactivity were similar to previous studies in that birds had significantly more Zenk-immunoreactive cells when listening to conspecific song compared to heterospecific song (Mello et al. 1992). As each starling
and canary song playback had equal ratios of noise:silence, I can assume that qualitative differences between the two species’ songs, rather than quantity of signal, was responsible for this effect. In the only study to date to examine how early-life stress affects Zenk-immunoreactivity in adult birds, Schmidt et al. (2013) found that song sparrows raised in stressful early-life conditions showed no difference in patterns of Zenk activation when listening to either their own species song or the similar songs of white-crowned sparrows (*Zonotrichia leucophrys*). Unlike the birds used by Schmidt et al. (2013), my birds reared in the treatment group did have significantly more Zenk-immunoreactive cells when listening to conspecific compared to heterospecific song; however, birds from the control group had significantly more Zenk-immunoreactive cells when listening to starling song than the treatment birds. There was no difference between either developmental treatment group when listening to canary song, which suggests that the developmental treatment in some manner dampens the selectivity of these auditory areas to respond to their own species’ song.

One auditory area that may be of particular importance for responding to variation in sexually relevant signals is NCM. Previous Zenk studies in starlings have found that females that were listening to song bouts that were longer (i.e., more attractive) had more activation in NCMv compared to females who listened to short song bouts (Gentner et al. 2001). Here, the control females were found to have more immunoreactivity in NCMv than treatment females in general. NCM plays a central role in the processing of auditory information and is part of auditory circuit that connects to areas within the telencephalon, such as the self of HVC and the cup anterior to RA (Mello et al. 1998). It also has a tonotopic organization, with certain areas being attuned to particular syllable types of
conspecific song (Ribeiro et al. 1998). Interestingly, NCM not only responds to the features of an acoustic sound but also appears to incorporate contextual information. The neural response to a previously habituated song can be increased if there is new information associated with the song, such as the song plays from another part of the cage or an additional stimulus is paired with the song such as a light (Kruse et al. 2004). Thus, NCM may play a crucial role in helping to direct the appropriate behaviour toward an auditory signal under different contexts which would be relevant to mate choice situations (Pinaud and Terleph 2008).

Lastly, there could be an inherited component to Zenk induction within the auditory areas measured here. Nest of origin explained a significant amount of the variation in Zenk expression, suggesting that there may be a genetic component to the variation in female’s response to a sexually selected trait. Females with a more discriminating auditory genotype may select males who are in better condition and could therefore drive sexual selection by having pairs of individuals with genotypes that would result in sexier sons and daughters with more discriminating preferences (Cotton et al. 2006). Understanding the genetic component to female preferences is a very new topic, but examining gene by environment interactions is a powerful way to address these questions.

4.4.3 Song-control system and its behavioural correlates

I did not find that the developmental treatment had long-term adverse effects on the volumes of three song-control system nuclei: HVC, RA and area X. Although there was no difference in volume between the developmental treatments, this does not rule out that other aspects of neuronal functioning were not affected (i.e., neuronal density,
connectivity, receptor density, etc.; Roth et al. 2010). My results are in accordance with
the only other studies to examine the effects of developmental stress on the female song-
control system. Juvenile female song sparrows euthanized immediately after the stressful
treatment ceased had smaller HVC compared to controls (MacDonald et al. 2006), but
volume deficits appear to lessen by adulthood (Schmidt et al. 2013). Although not
significant, the last term to leave the model ($P = 0.077$) was whether birds participated in
the cognitive treatment: birds that did had smaller song-control volumes than birds that
did not. I measured these areas several months after the cognitive experiments ended, but
it would suggest that the reduction in volume to areas within the song-control system may
be apparent while an environmental stress is present or shortly after, but that the song-
control system is relatively robust to recovering volume losses if given at the very least
several months of rehabilitation. The volume of nuclei within the song-control system of
male starlings is known to fluctuate greatly as a result of age, seasonal changes,
circulating hormones, and the amount of song a male produces (Bernard et al. 1996;
Sartor and Ball 2005; Ball and Balthazart 2010). The song-control system exhibits
tremendous neurogenesis and plasticity and therefore its condition may be more
indicative of recent conditions experienced and not solely early-life environments
(Newman et al. 2010; Wada et al. 2013).

Although developmental stress did not affect HVC volume, HVC volume was still
correlated with a bird’s preference towards conspecific song. I found that the size of
HVC was positively related to a female’s preference towards listening to conspecific
song compared to heterospecific song as measured in the behavioural assay. A similar
relationship has been observed before in both female starlings and canaries (Leitner and
Catchpole 2002; Riters and Teague 2003). In canaries, HVC is necessary for the perception of conspecific song and neurons within HVC will fire more rapidly when sexy syllables are played (Brenowitz 1991; Del Negro et al. 2000). My results from the present study offer further support that HVC plays a role in female song perception and discrimination.

4.4.4 Conclusions

When there are benefits to possessing a strong preference function then there will be a cost to pay in order to possess it (Cotton et al. 2006). In starlings, female song preferences are condition-dependent – females in the control treatment have behavioural and neural responses that would result in heightened mate evaluation abilities over their developmentally stressed counterparts. And ultimately, by paying the costs to develop these abilities these females will locate a desirable mate faster, which nets them benefits in terms of increased reproductive success. This study is able to address some proximate constraints females may have on their ability to process and discriminate song signals of varying biological relevance. Female starlings subjected to stressful early-life conditions had a more muted neural response to conspecific song and this deficit was mirrored by the lack of preference towards actively choosing to listen to conspecific song. Females that were developmentally stressed, even though they may have had a greater neuronal response to their own species song, were unable to integrate information about a song stimulus with contextual information (i.e., associating a song with a perch location). This study’s results are in accordance with previous work on male songbirds that show that developmental stress can have adverse effects on the female songbirds brain and behaviour.
4.5 References


Schmidt KL, MacDougall-Shackleton EA, MacDougall-Shackleton SA (2012) Developmental stress has sex-specific effects on nestling growth and adult metabolic
West BT, Welch KB, Galecki AT (2007) Linear mixed models: a practical guide to using statistical software. Taylor and Francis Group, Boca Raton (FL)
Chapter 5

5 Effects of developmental stress on song bout length and neural development are temporary in European starlings (*Sturnus vulgaris*)

5.1 Introduction

Birdsong is a complex, learned vocalization that is supported by an interconnected series of nuclei collectively referred to as the song-control system. Song learning begins early in development, wherein a young songbird must first be exposed to adult tutor song and then practice singing while the song-control system is plastic and open to sensory input (Catchpole and Slater 2008). Both within and between species, positive correlations have been noted between song nuclei volumes (HVC [proper name] and RA [robust nucleus of the arcopallium]) and measures of song quality (DeVoogd et al. 1993; Garamszegi and Eens, 2004). As well, females often prefer songs that are more complex, greater in length, and/or match the local dialect (Gil and Gahr 2002; Nowicki and Searcy 2005). Females that select males with high song quality may thus also be selecting a mate with enhanced brain function and connectivity, which in turn may be indicative of direct (e.g. material benefits) or indirect (i.e., genetic) benefits she may receive for herself and future offspring (Searcy and Andersson 1986; Gil and Gahr 2002; DeVoogd 2004).

Song learning and development of the song-control system coincides with rapid post-natal growth, wherein songbirds are highly susceptible to periods of nutritional deprivation and disease. The *developmental stress hypothesis* proposes that song is maintained as an honest indicator of male quality as only males of high phenotypic and/or
genotypic quality would be able to afford the costs to develop the song-control system during challenging environmental conditions (Nowicki et al. 1998). Males of inferior quality would trade-off costly neural development of the song-control system in exchange for short-term benefits gained by maintaining somatic growth (Schew and Ricklefs 1998). Assessments of the developmental stress hypothesis, employing a number of species and developmental stressors, generally supports the idea that stressors experienced during early-life result in poorer song-control system development, song quality, and other aspects of the phenotype (reviewed in MacDougall-Shackleton and Spencer 2012; Spencer and MacDougall-Shackleton 2011).

Quantifying changes in the volume of individual song-control nuclei has been the principal metric used to evaluate the neural effects of developmental stressors. HVC appears to be highly susceptible to developmental stress. Studies inducing food-restriction, increased corticosterone levels, or exposure to illness (i.e., avian malaria), have found reductions in HVC volume (Buchanan et al. 2004; Spencer et al. 2005; MacDonald et al. 2006), fewer androgen receptors (Buchanan et al. 2004), and decreased rates of neurogenesis (Honarmand et al. 2015). RA may be less susceptible, however smaller volumes have been observed in emberizid sparrows reared on food-restricted diets compared to controls (MacDonald et al. 2006; Nowicki et al. 2002; Schmidt et al. 2013). It should be noted that not all studies detect neural effects, but those studies with null effects on the song-control system have also failed to find an effect on song quality (e.g., Gil et al. 2006).

As the song-control system is a series of interconnected nuclei (Vates et al. 1997), connectivity between nuclei could also be affected by developmental stressors. HVC
sends efferent projections to RA, referred to as the HVC-to-RA tract, which constitutes the motor production pathway (caudal motor path) of the song-control system (Nottebohm et al. 1976; Simpson and Vicario 1990). As part of the anterior forebrain path, HVC sends efferent projections to the striatal nucleus area X via the lamina mesopallium ventralis tract (LMV; formerly lamina mesopallii, LaM; Jarvis et al. 2013). This tract is involved in the acquisition and maintenance of song (Nottebohm et al. 1976). Nuclei of the song-control system and the tracts between them are myelinated, and changes in myelination within them are associated with behavioural changes in song production (von Bohlen und Halbach and Nixdorf-Bergweiler 2004; De Groof et al. 2008). Myelin can reduce the metabolic costs of neuronal firing and improve neuronal transmission between nuclei (Hartline 2008). Yet, myelin is metabolically expensive to produce and is highly susceptible to the effects of oxidative stress (Bradl and Lassmann 2010). To date, no study has examined the effects developmental stress has on myelination within the tracts of the song-control system.

Substantial research has documented how developmental stress can affect song quality and the song-control system, but only until the first breeding season. This is in part because the majority of species assessed are unable to alter their song repertoire once they are reproductively mature (i.e., they are closed-ended song learners). Yet, some species are capable of learning new songs throughout their lifespan (i.e., open-ended learners; Beecher and Brenowitz 2005). The European starling (Sturnus vulgaris) is an excellent model to study the long-term effects of developmental stressors on song quality and the song-control system. It has been empirically demonstrated that starlings are open-ended learners and that female starlings strongly prefer males with more complex songs.
(as measured by repertoire size and song bout length: Chaiken et al. 1994; Eens et al. 1991; Gentner and Hulse 2000). Starling song complexity is indicative of various measures of quality, such as enhanced immune function, higher dominance ranking and greater spatial learning ability (Duffy and Ball 2002; Spencer et al. 2004; Farrell et al. 2011). In the wild, males with longer songs find a mate faster and fledge more young (Eens et al. 1991). The relationship between male condition and song quality is thus well established in this species. Developmental changes within the song-control system have also been documented, with starlings increasing the volumes of HVC and RA, although not area X, with age; HVC and RA size are larger in older males than in 1-year old males (Bernard et al. 1996). Myelination of the song-control system is known to change with age and season, corresponding to behavioural changes in song learning and production (Herrmann and Bischof 1986; Stocker et al. 1994; De Groof et al. 2008). However, the developmental changes in myelination of the song-control system have yet to be studied in an open-ended learner across multiple breeding seasons. Learning new songs in adulthood may be facilitated by increased myelination within tracts that connect song-control nuclei. Open-ended learners, due to their altered neuronal plasticity (Brenowitz 2004), could recuperate from a poor developmental start. Relative to closed-ended learners, developmental stress may be less debilitating on lifetime reproductive success.

The aim of this chapter is to investigate how developmental stressors affect song behaviour and neural development at different developmental stages in a songbird with open-ended song learning. Using a cross-sectional design, I examined the effects of an unpredictable food supply enforced in the juvenile period (i.e., ~35 to 115 days of age) on starlings at 4, 12, and 24 months of age. My first objective was to assess if this
developmental stressor known to reduce song quality for yearling males (i.e., 12 months of age) would continue to have effects into adulthood (i.e., 24 months of age). My second objective was to assess the effects on the development of the song-control system following the immediate termination of the treatment when birds were juveniles (i.e., 4 months of age), several months later as yearlings and over a year later as adults. To address this, I measured the volume of three nuclei: HVC, RA, and area X in Nissl-stained tissue. I also measured the extent of myelination of the HVC-to-RA tract and the LMV tract. To measure myelination I used immunohistochemistry to label myelin basic protein (MBP). MBP is a marker of myelin within the central nervous systems that specifically binds to peptides located in the myelin cytoplasmic membrane. Lastly, my third objective was to document age-related differences in behaviour and the brain. I predicted that developmental stressors would exert their effects on song behaviour in the first breeding season, but this effect would not carry over to the second breeding season. Similarly, I predicted that the strongest effects on neural development of the song-control system would be apparent earlier in development, but these effects would dissipate by the second breeding season. And overall, I predicted song production, song-control nuclei volumes, and myelination of the song-control system would increase with age.

5.2 Methods

5.2.1 Subjects and Treatment

Subjects were 62 male starlings (Table 5-1). Juvenile (4-month old) and yearling (12-month old) males were the same males subjects as in Chapters 2 and 3. Adult (24-month old) males studied here were captured as nestlings and juveniles around London, Ontario (42°98’ N, 81°25’ W) in 2009 and were the same subjects as in Farrell et al.
(2011). Birds caught in 2009 followed a similar protocol as outlined in Chapter 2, but
with some variations outlined below.

Table 5-1 Sample size by developmental treatment and age

<table>
<thead>
<tr>
<th>Developmental Treatment</th>
<th>Juvenile 4-month old</th>
<th>Age</th>
<th>Yearling 12-month old</th>
<th>Adult 24-month old</th>
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<tr>
<td>Treatment</td>
<td>8</td>
<td>8</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>9</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

5.2.1.1 2009 Cohort

In May –July 2009, I captured 29 male starlings as either nestlings (i.e., younger than 21 d; n = 14) or fledged juveniles (i.e., older than 21 d; n = 15). Once in the laboratory, I randomly assigned birds to either treatment or control groups. Briefly, nestling-caught birds from the control conditions were hand-fed *ad libitum* a nestling diet [chick starter, wheat germ, carrots, vitamin, hardboiled egg, grit, and vitamins (Prime avian vitamin supplement, Rolf C. Hagen Inc, Montreal, QC, Canada)], while their food-restricted siblings were fed 65% of the average amount of food eaten by controls through calibrated plastic syringes. Once feeding independently, all birds were housed by treatment condition in outdoor aviaries (358cm X 212cm X 216cm). Once eating independently, the nestling-caught birds continued their developmental treatment and were put on the same regime as the juvenile-caught birds. During their juvenile phase, control birds were housed together in an aviary and had *ad libitum* access to food (chick starter), while food-restricted birds had their food removed for a 4 h interval daily at a random time between 09:00-17:00 h until treatment ceased at approximately 90 days of age (as per Buchanan et al., 2003). After treatment ended in August, I housed all birds
indoors in individual cages (76cm X 46cm X 45cm) with *ad libitum* food and water. Between 4 to 10 months of age, birds were assessed on a variety of behavioural tasks (Farrell et al 2011). At approximately 2 years of age, birds’ song performance was assessed (explained below). After song performance was assessed at 2 years of age, birds were euthanized and their brains were collected for further analyses (explained below). Birds from the 2009 cohort compromise the group herein referred to as ‘adults’ for the song and neural analyses.

**5.2.1.2 2012 Cohort**

Birds caught in 2012 were the same subjects from Chapter 2. At the end of the treatment period, when birds were about 4 months of age, a subset of birds from both control and treatment groups were euthanized and their brains were collected. These birds represent the birds in the brain analysis herein referred to as ‘juveniles’. The remaining birds in the control and treatment groups had their song performance assessed and their brains collected at approximately 12 months of age. These birds are herein referred to in the song and brain analyses as ‘yearlings’. A subset of the yearling birds were assessed on a series of auditory learning tasks from approximately 6 to 11 months of age as outlined in Chapter 3. To ensue high motivation, birds were food-restricted to 85-90% of their *ad libitum* weight for the duration of this testing. This period of food-restriction was an unintended additional stressor and had documented effects on endocrine function (Chapter 2; Farrell et al. 2015a), and neural and behavioural measures in female starlings (Chapter 4; Farrell et al. 2015b). Therefore, I include this factor (i.e., cognitive treatment) in the statistical analyses assessing song performance and neural measurements for this age group.
The 2012 cohort differs from the 2009 cohort in several respects. (1) All birds in the 2012 cohort were caught as nestlings and the age they started the developmental treatment was known. Juvenile caught birds in the 2009 cohort started the developmental treatment at an estimated age that was several weeks older than the nestling caught birds (Farrell et al. 2011). (2) Birds in the 2012 cohort were individually housed indoors for the duration of the study. The 2009 cohort had a different social dynamic, as birds were group-housed by treatment condition in outdoor aviaries for the duration of the treatment period. (3) Birds in the 2012 cohort were on the developmental treatment until 115 days of age, which was 25 days longer than the 2009 cohort. And lastly (4), a subset of birds in the 2012 cohort experienced additional environmental food restrictions as a result of the cognitive experiments conducted in Chapter 3.

5.2.2 Song Recording and Analysis

I recorded the singing performance of yearling and adult males in a courtship condition in 2013 and 2011, respectively. Birds were in breeding condition during the recordings and were recorded after being exposed to the natural photoperiod (≥ 15 hours of light) of London, Ontario in June-July for several weeks (range: 2-5 weeks). The courtship song procedure was the same across both years and was identical to the one used previously with adults as yearlings during their first breeding season in 2010 (Farrell et al. 2011). On the day of recording, I placed a male in a recording cage in the morning. I recorded birds for two 1 h sessions within the 24 h period they were housed in the recording cage. The first recording session took place the afternoon the bird entered the recording cage between 15:00-17:00 h. The second recording session took place the following morning between 09:00-11:00 h. To stimulate the male to sing, the recording
cage contained a female starling (the same female across both years), a nestbox, a water bath, and \textit{ad libitum} food and water. The recording cage was adjacent to cages with small groups of males, so while the male being recorded was physically isolated, he was in acoustic and visual contact with other males. Behind a one-way mirror and blind to treatment group, I scored singing behavior with J-watcher software (Blumstein et al. 2006) using (1) posture and stance cues (Feare 1984) and (2) real-time auditory signals being transmitted indoors via a Marantz PMD 671 recorder attached to a Sennheiser ME62 microphone mounted in a parabola outside the recording cage. Average song bout length (i.e., total duration of singing / number of song bouts) was calculated for each individual as a measure of song complexity. As per Eens (1997), a song bout was defined as continuous song greater than 5 s in duration with no longer than a 1 s pause. Starling song is very complex song and as multiple males were singing concurrently during the recording sessions, explicit analysis of an individual’s repertoire size was not possible when visualizing spectrograms. However, repertoire size and mean song bout length are strongly correlated and mean song bout length is a commonly used index of song complexity in this species (Eens 1997; Buchanan et al. 2003; Farrell et al. 2011).

When the song recordings were complete, the target male was moved into an adjacent aviary with \textit{ad libitum} food and water that provided visual and auditory contact with other birds. Later that day, at 13:00, I removed the bird from the aviary and rapidly overdosed him on isoflurane. I then decapitated the bird, removed the brain, froze it on powdered dry ice, and stored the tissue at \(-80\, ^\circ\text{C}\) until further analysis. For the 2012 cohort, birds completed a 1 h GnRH challenge (see Chapter 2; Farrell et al. 2015a) prior to being euthanized.
5.2.3 Nissl histology and quantification

I sectioned brains using a cryostat, cutting along the sagittal plane in 20 µm sections. I thaw-mounted every fifth section on to electrostatically treated microscope slides (VWR VistaVison ™ Histobond ®), alternating sections into series for Nissl histology and MBP immunohistochemistry (explained below). For the Nissl series, I immersed slides in 4% paraformaldehyde for 10 min, rinsed them in 0.1 M phosphate buffered saline (PBS; pH 7.4), and left them to air-dry overnight before processing them the following day. For the MBP series, I left slides to air-dry overnight and then stored them at -80 °C until further analysis.

The sections for the Nissl series were stained with thionin, serially dehydrated in graded ethanol, cleared in a solvent (Harleco Neoclear) and protected by affixing a coverslip with Permount (Fisher Scientific). I quantified the volume of the three song-control nuclei of interest: HVC, area X, and RA. I calculated the volumes by taking images from every section that contained the region of interest using a Spot Idea 5-megapixel digital camera (Diagnostics Instruments) connected to a Zeiss Axiophot microscope. I then traced the outlines of each nucleus using ImageJ (National Institutes of Health, Bethesda, MD, U.S.A.) and then estimated the total volume of the nucleus by combining cross-sectional areas and the sampling intervals (5 x 20 µm = 100 µm) using the formula for the volume of a frustum (truncated cone). To estimate total brain size (telencephalon volume), microscope slides were scanned at 1200 dpi on a flatbed scanner with a transparency adapter. Using ImageJ I traced the telencephalon in every 40th section (800 µm sampling interval) and using the formula for the volume of a frustum I estimated total telencephalon volume.
5.2.4 MBP immunohistochemistry

The MBP immunohistochemistry was piloted on two adult male starlings not part of this study. The HVC-to-RA fiber tract had the most intense myelin staining when the cross-sectional area of HVC was largest and RA was present in the same section. Using these criteria, I used the corresponding sections labeled with Nissl to select approximately 6-15 sections per bird for MBP immunohistochemistry processing. Fewer sections were processed for younger birds as the song-control nuclei were significantly smaller in volume and did not traverse as many sections. The LMV tract traverses a larger mediolateral distance than the HVC-to-RA tract and was present in all sections selected.

I ran immunohistochemistry in six runs, counterbalanced across treatment conditions and age at time of brain collection. I adapted an established MBP protocol for free-floating fixed sections (A. Pirano, unpublished results) to be used with fresh frozen tissue mounted directly onto slides (Sundquist and Nisenbaum 2005). Prior to starting the protocol, all slides were thawed to room temperature and I encircled the tissue with a hydrophobic marking pen (Liquid Blocker; Daido Sangyo Co, Ltd., Tokyo, Japan) to prevent reagent run-off during the incubations. All incubations described below were done on horizontal slides inside a custom humid chamber by covering each slide in 250 µL of reagent, or until all tissue was covered. After the blocking steps were complete, I tipped the slide over and pipetted liquid from a tissue free area of the slide to remove the reagent.

First, slides were immersed in 4% paraformaldehyde for 10 min and then rinsed in 0.1 M PBS. Then, slides were washed four times in 0.1% Triton in PBS (PBST), followed
by one wash in 0.1 M PBS. I then incubated slides for a 15 min incubation in 0.5% H₂O₂ to eliminate endogenous peroxidase. I then washed the tissue three times in 0.1% PBST prior to a 1 h blocking incubation in 10% normal goat serum (Vector, Burlingame, CA) diluted in 0.3% PBST. Sections were then incubated at room temperature for 20 h in the primary antibody (anti-myelin basic protein antibody [12], ab7349; Abcam, Cambridge, UK) diluted 1:250 in 10% normal goat serum diluted in 0.3% PBST. I then washed sections three times in 0.1% PBST and followed this with a 1 h incubation in biotinylated secondary antibody (biotin-conjugated anti-rabbit IgG made in goat, Vector) diluted 1:400 in 10% normal goat serum diluted in 0.3% PBST. Following three washes in 0.1% PBST, sections were incubated in avidin-biotin horseradish-peroxidase complex (Vectastain Elite ABC kit, Vector Labs) for 1 h and then washed four times in 0.1% PBST. I then visualized sections with diaminobenzidine solution (SIGMAFAST™ DAB with Metal Enhancer, Sigma-Aldrich Co, St. Louis, MO) and washed them 5 times in 0.1 M PBS. Last, slides were serially dehydrated with graded ethanols, cleared in a solvent (Harleco Neo-Clear®, EMD Chemicals, Missassauga, ON, Canada) and protected by affixing a coverslip with Permount (Fisher Scientific, Fair Lawn, NJ).

5.2.5 MBP quantification

An observer, blind to treatment and age, captured images through a 1.25x objective lens using a Spot Idea 5-megapixel digital camera (Diagnostics Instruments) connected to a Zeiss Axiophot microscope. I selected the section that had the highest proportion of myelin staining for the HVC-to-RA tract and then measured the two sections on either side. Therefore, I measured five sections (spaced every 100 µm) for a
total sampling area that spanned 500 µm wide mediolaterally. The LMV tract was quite pronounced in all sections, therefore I measured the LMV tract in the same five sections.

Using ImageJ, I converted all images to 16-bit grayscale and these images were used to count the number of pixels with an optical density that was above a threshold value using the threshold feature (see Figure 5-1). Using the straight-line tool in ImageJ, I traced a line from the edge of the middle of HVC to the edge of RA such that the line bisected the HVC-to-RA tract and I recorded the coordinates of the midpoint of this line (Figure 5-1b). As there was variability in the intensity of the background staining, I manually set the threshold for each image such that a blind observer agreed that the highlighted pixels corresponded to visible myelin tracts (Figure 5-1c and 5-1d). Then, I specified a sampling box (500 x 500 pixels) centered at the coordinates of the line drawn in the previous step (Figure 5-1e). As the LMV tract is thick and compact, I selected the midpoint of the tract and recorded the coordinates of the midpoint. Then, I specified a sampling box (250 x 750 pixels) centered on the recorded coordinates. Within each sampling box, I used the analyze particle command, with the minimum object size set at 30 pixels to preclude measuring dust artifacts. For both the HVC-to-RA tract and the LMV tract, I recorded the total proportion of labeled pixels (% area cover), the average size (in pixels) of continuously labeled myelin fibers, and the total number of individual stained fibers within the tract. Note that use of the term fibers as defined here is measuring clusters of several fibers, and not individually distinct myelinated axons.
Figure 5-1 Sagittal section of the male starling brain indicating the regions, and steps taken in Image J, where MBP immunoreactivity was quantified. Ventral is down and caudal is right in all photos. (A) Boxes are highlighted around the LMV and HVC-to-RA tract to show the approximate areas sampled for each tract. (B-E) The steps to quantify myelin within the HVC-to-RA tract are demonstrated. (B) A line is drawn from HVC to RA that bisects the tract and the coordinates of the midpoint of the line are recorded. (C-D) Using a manual threshold, the image is then converted into a black and white image. (E) Lastly, the sampling window is centered upon the coordinates recorded in panel B.

5.2.6 Data and Statistical Analysis

All data were analyzed using linear mixed models. As the 2009 and 2012 cohorts differed on a variety of aspects (outlined in section 5.2.1.2 above), I did not analyze the
interaction between developmental treatment and age. Also, there were factors specific to each age group that made examining higher-order interactions with age and developmental treatment not possible. Therefore, I first conducted statistical models to assess the general effect of age on song bout length, song-control nuclei volumes, and myelination of the HVC-to-RA tract and LMV tract. Then, within each age group, I assessed the effects of developmental treatment, and tailored each model by adding additional fixed effects of interest (and any of their higher-order interactions with developmental treatment) and covariates of interest specific to that age group. Random effects of nest of origin and hemisphere analyzed were first included in all applicable models. However, neither effect contributed significantly to any model and both were removed from the final analysis.

For song production, I ran separate analyses using mean song bout length as the dependent variable. All models for each age group included the fixed effect of developmental treatment. I then included additional fixed effects specific to each age group. The model for the yearling birds included a covariate of the number of days birds were food-restricted as part of the cognitive experiments conducted in chapter 3. This was entered as a covariate, as opposed to a fixed effect, due to the small sample size (three males from the developmental treatment were excluded from the analysis as they didn’t sing). The model for the adult birds included the fixed effect of the age at which birds were captured and its interaction with developmental treatment.

For each song-control nucleus, I ran separate analyses using the volume of each nucleus (HVC, RA, or area X) as the dependent variable. All models for each age group included the fixed effect of developmental treatment and telencephalon volume (minus
the volume of the nucleus of interest) as a covariate. For example, analyses of nucleus RA would use each subject’s telencephalon volume minus the volume of their RA as a covariate. Additional age-specific effects were also included. All models for yearling birds included the fixed effect of participation in the cognitive experiments and its interaction with developmental treatment. All models for adult birds included the age at which birds were captured and its interaction with developmental treatment.

For the MBP immunoreactivity of the HVC-to-RA tract and LMV tract, I ran separate analyses on each dependent variable. All models for each age group included the fixed effect of developmental treatment and HVC volume as a covariate. Additional age-specific effects were also included and were the same as those specified above for the models analyzing song-control nuclei.

I initially constructed all models as fully loaded models (all predictors and interactions included). Then I used a top-down approach by removing non-significant predictors to maintain parsimony and improve model fit (Chapter 2; West et al. 2007). Model fit was assessed using likelihood ratio tests. All significant results reported here are the minimal adequate models with restricted maximum likelihood estimation. Values for non-significant effects are the values from the full model prior to being removed. All post hoc tests were carried out using Bonferroni corrections and I checked the residuals of each model against a normal distribution and found no violations of normality. All data are presented as means ± SE, adjusted for significant covariates where applicable. All analyses were conducted in IBM SPSS Statistics (v. 21).
5.3 Results

5.3.1 Song data

5.3.1.1 All ages

Average mean song bout length increases significantly with age in starlings ($F_{1,40} = 28.323, P < 0.00001$). Adult birds had significantly longer mean song bouts than yearling birds (Figure 5-2).

![Figure 5-2 Mean song bout length (s) by age and treatment group. Yearlings (2012 cohort) sang significantly shorter songs than the adults (2009 cohort). There was no difference in mean song bout length between control (white bars) and treatment (black bars) birds within each developmental age. **** $P < 0.00001$](image)

5.3.1.2 Yearling

Unlike previous studies (Farrell et al., 2011), developmental treatment did not affect mean song bout length of yearling males ($F_{1,14} = 2.182, P = 0.162$; Figure 5-2). However, three males (all reared in the treatment group) did not sing in my courtship condition. Two of these males also participated in the cognitive treatment. Participation
in the cognitive treatment was not a significant covariate in mean song bout length (F\textsubscript{1,14} = 2.124, P = 0.167).

### 5.3.1.3 Adult

While developmental treatment reduced mean song bout length in the first breeding season (data not shown; see Farrell et al. 2011), there was no long-term effect on mean song bout length as measured in the second breeding season (F\textsubscript{1,28} = 0.186, P = 0.670; Figure 5-2). This indicates that the song impairment noted in the first breeding season for birds reared in the treatment group was temporary and birds were able to match controls after another year of rehabilitation. Age at which birds were captured did not have a significant effect (F\textsubscript{1,28} = 0.022, P = 0.884), nor did it interact significantly with developmental treatment (F\textsubscript{1,28} = 1.194, P = 0.284).

### 5.3.2 Song-control nuclei volumes

#### 5.3.2.1 All ages

The volumes of the song-control regions increased significantly with age (Figure 5-3). HVC, RA, and area X volumes were smallest for juvenile birds, grew to an intermediate size for yearling birds, and were largest in adult birds (HVC: F\textsubscript{2,56} = 41.347, \(P < 0.0001\); RA: F\textsubscript{2,55} = 56.955, \(P < 0.0001\); Area X: F\textsubscript{2,55} = 48.570, \(P < 0.0001\); Figure 5-4a). Telencephalon size was a significant positive covariate in all of the analyses (HVC: F\textsubscript{1,56} = 9.337, \(P < 0.01\); RA: F\textsubscript{1,55} = 19.551, \(P < 0.0001\); Area X: F\textsubscript{1,55} = 19.808, \(P < 0.0001\)). Thus, birds with larger telencephalons did tend to have larger song-control nuclei.

I also analyzed the difference in telencephalon size across the ages, while controlling for mass at the time of brain collection. Age was a significant factor (Age:
Figure 5-3 Photomicrographs of Nissl stained sagittal sections containing HVC (top), RA (middle), and area X (bottom) from representative control juvenile (left column), yearling (middle column), and adult (right column) males. Ventral is down and caudal is right in all photos. Black arrows indicate nuclear borders. Scale bar = 500 µm

\[ F_{2,58} = 20.160, P < 0.000001 \] and mass at time of death was a significant positive covariate \( (F_{1,58} = 5.472, P = 0.023) \). Juvenile and yearling birds did not differ in
Figure 5-4 Developmental changes in the song-control system across juvenile (black bars), yearling (gray bars), and adult starlings (white bars). (A) Volumes of all song-control nuclei increase significantly with age. (B) Proportion of myelination of the HVC-to-RA tract increases drastically from juvenile to yearlings (left y-axis). The average size of each myelinated tract increases significantly from juvenile to adult (right y-axis). (C) Proportion of myelination of the LMV tract increases drastically from juvenile to yearlings (left y-axis), but there is no difference in average size of each myelinated tract (right y-axis). * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$
telencephalon volume \( (P = 0.873) \), but adult birds had significantly smaller telencephalon volumes than juveniles \( (P < 0.00001) \) and yearlings \( (P < 0.001) \). As telencephalon volume varies across ages, this was an additional reason to perform individual statistical models for each age group individually to compare effects of the developmental treatment.

### 5.3.2.2 Juvenile

Developmental treatment had the largest effect on song-control nuclei volumes immediately after termination of the treatment (Figure 5-5a). Treatment birds had significantly smaller HVC volumes compared to control birds (Table 2). While not significant, developmental treatment remained in the final model for RA volume, with treatment birds having smaller RA volumes compared to control birds. There was no difference in volume between treatment groups for area X. Birds with larger telencephalon volumes had larger RA and area X volumes, but there was no relationship between telencephalon and HVC volume (Table 2).

### 5.3.2.3 Yearling

The effects of the developmental treatment changed with age. Compared to juveniles, there was no difference in HVC and RA volumes between the treatment groups (Figure 5-5b; Table 3). However, treatment birds had significantly smaller area X volumes than control birds (Figure 5-5b). Participation in the cognitive treatment did not affect the volume of HVC, RA or area X. For all three nuclei, telencephalon size was not a significant covariate and there were no significant interactions between the developmental treatment and cognitive treatment (Table 3).
Figure 5-5 Effect of the developmental treatment (control: white bars; treatment: black bars) on the volumes of song-control nuclei across (a) juvenile, (b) yearling, and (c) adult starlings. (A) In juveniles, HVC and RA are smaller in treatment males compared to controls. (B) In yearlings, area X is smaller in treatment males compared to controls. (B) In adulthood, there is no difference in song-control nuclei volumes across treatment and control birds. * P < 0.05, + P < 0.06
Table 5-2 Results of linear mixed models for measures of song-control nuclei volumes, myelination of the HVC-to-RA tract, and the LMV tract for juvenile birds (age 4 months).

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<thead>
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<th>Variable</th>
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<td></td>
<td>HVC volume</td>
<td>0.058</td>
<td>1, 13</td>
<td>0.813</td>
</tr>
<tr>
<td>LMV average size of tract</td>
<td>Treatment</td>
<td>0.247</td>
<td>1, 15</td>
<td>0.626</td>
</tr>
<tr>
<td></td>
<td>HVC volume</td>
<td>0.000</td>
<td>1, 15</td>
<td>0.998</td>
</tr>
<tr>
<td>LMV no. of stained sections</td>
<td>Treatment</td>
<td>0.661</td>
<td>1, 15</td>
<td>0.429</td>
</tr>
<tr>
<td></td>
<td>HVC volume</td>
<td>0.412</td>
<td>1, 15</td>
<td>0.531</td>
</tr>
</tbody>
</table>

Covariates are in *italics*. Variables that were significant (α < 0.05) are highlighted in **bold**.

* Variables that were not significant, but remained in the final model.

Nest of origin was not significant in any of the models.
Table 5-3 Results of the linear mixed models for song-control nuclei volumes, myelination of the HVC-to-RA tract, and the LMV tract for yearling birds (age 12 months).

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Variable</th>
<th>$F$-ratio</th>
<th>df</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
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<td>HVC volume</td>
<td>Treatment</td>
<td>0.404</td>
<td>1, 17</td>
<td>0.533</td>
</tr>
<tr>
<td></td>
<td>Early adult food-restriction</td>
<td>0.097</td>
<td>1, 17</td>
<td>0.759</td>
</tr>
<tr>
<td></td>
<td>Treatment X Early adult food-restriction</td>
<td>0.751</td>
<td>1, 17</td>
<td>0.398</td>
</tr>
<tr>
<td></td>
<td><em>Telencephalon</em></td>
<td>0.995</td>
<td>1, 17</td>
<td>0.332</td>
</tr>
<tr>
<td>RA volume</td>
<td>Treatment</td>
<td>0.059</td>
<td>1, 17</td>
<td>0.811</td>
</tr>
<tr>
<td></td>
<td>Early adult food-restriction</td>
<td>0.672</td>
<td>1, 17</td>
<td>0.424</td>
</tr>
<tr>
<td></td>
<td>Treatment X Early adult food-restriction</td>
<td>0.001</td>
<td>1, 17</td>
<td>0.974</td>
</tr>
<tr>
<td></td>
<td><em>Telencephalon</em></td>
<td>1.624</td>
<td>1, 17</td>
<td>0.220</td>
</tr>
<tr>
<td>Area X volume</td>
<td><strong>Treatment</strong></td>
<td>5.054</td>
<td>1, 15</td>
<td>0.040</td>
</tr>
<tr>
<td></td>
<td>Early adult food-restriction</td>
<td>3.189</td>
<td>1, 17</td>
<td>0.092</td>
</tr>
<tr>
<td></td>
<td>Treatment X Early adult food-restriction</td>
<td>2.739</td>
<td>1, 17</td>
<td>0.116</td>
</tr>
<tr>
<td></td>
<td><em>Telencephalon</em></td>
<td>0.064</td>
<td>1, 17</td>
<td>0.804</td>
</tr>
<tr>
<td>HVC-to-RA % area of MBP</td>
<td>Treatment</td>
<td>0.593</td>
<td>1, 17</td>
<td>0.452</td>
</tr>
<tr>
<td></td>
<td>Early adult food-restriction</td>
<td>4.875</td>
<td>1, 14</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>Treatment X Early adult food-restriction</td>
<td>0.031</td>
<td>1, 17</td>
<td>0.862</td>
</tr>
<tr>
<td></td>
<td><em>HVC volume</em></td>
<td>4.439</td>
<td>1, 14</td>
<td>0.054*</td>
</tr>
<tr>
<td>HVC-to-RA average size of tract</td>
<td>Treatment</td>
<td>4.420</td>
<td>1, 13</td>
<td>0.056*</td>
</tr>
<tr>
<td></td>
<td>Early adult food-restriction</td>
<td>10.716</td>
<td>1, 13</td>
<td>0.006</td>
</tr>
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<td></td>
<td>Treatment X Early adult food-restriction</td>
<td>3.126</td>
<td>1, 17</td>
<td>0.095</td>
</tr>
<tr>
<td></td>
<td><em>HVC volume</em></td>
<td>24.546</td>
<td>1, 13</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HVC-to-RA no. of stained sections</td>
<td>Treatment</td>
<td>2.438</td>
<td>1, 17</td>
<td>0.137</td>
</tr>
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<td></td>
<td>Early adult food-restriction</td>
<td>1.350</td>
<td>1, 17</td>
<td>0.261</td>
</tr>
<tr>
<td></td>
<td>Treatment X Early adult food-restriction</td>
<td>4.086</td>
<td>1, 17</td>
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</tr>
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<td><em>HVC volume</em></td>
<td>2.707</td>
<td>1, 17</td>
<td>0.118</td>
</tr>
<tr>
<td>LMV % area of MBP</td>
<td>Treatment</td>
<td>0.614</td>
<td>1, 17</td>
<td>0.444</td>
</tr>
<tr>
<td></td>
<td>Early adult food-restriction</td>
<td>0.074</td>
<td>1, 17</td>
<td>0.789</td>
</tr>
<tr>
<td></td>
<td>Treatment X Early adult food-restriction</td>
<td>1.590</td>
<td>1, 17</td>
<td>0.224</td>
</tr>
<tr>
<td></td>
<td><em>HVC volume</em></td>
<td>0.077</td>
<td>1, 17</td>
<td>0.785</td>
</tr>
</tbody>
</table>
5.3.2.4 Adult

For all three nuclei, there was no difference between developmental treatment groups, age at which birds were captured, or their interaction (Figure 5-5c; Table 4).

Telencephalon volume was a significant positive covariate for the volumes of HVC, RA and area X (Table 4).

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Variable</th>
<th>F-ratio</th>
<th>df</th>
<th>P value</th>
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</thead>
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<td>Treatment</td>
<td>0.461</td>
<td>1, 27</td>
<td>0.503</td>
</tr>
<tr>
<td></td>
<td>Age at capture</td>
<td>0.002</td>
<td>1, 27</td>
<td>0.965</td>
</tr>
<tr>
<td></td>
<td>Treatment X Age at capture</td>
<td>0.186</td>
<td>1, 28</td>
<td>0.669</td>
</tr>
<tr>
<td></td>
<td>Telencephalon</td>
<td>6.642</td>
<td>1, 26</td>
<td>0.016</td>
</tr>
<tr>
<td>RA volume</td>
<td>Treatment</td>
<td>0.018</td>
<td>1, 28</td>
<td>0.895</td>
</tr>
<tr>
<td></td>
<td>Age at capture</td>
<td>0.001</td>
<td>1, 28</td>
<td>0.979</td>
</tr>
<tr>
<td></td>
<td>Treatment X Age at capture</td>
<td>0.062</td>
<td>1, 27</td>
<td>0.804</td>
</tr>
<tr>
<td></td>
<td>Telencephalon</td>
<td>13.487</td>
<td>1, 25</td>
<td>0.001</td>
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<tr>
<td>Area X volume</td>
<td>Treatment</td>
<td>0.058</td>
<td>1, 28</td>
<td>0.812</td>
</tr>
<tr>
<td></td>
<td>Age at capture</td>
<td>0.019</td>
<td>1, 28</td>
<td>0.890</td>
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Covariates are in *italics*. Variables that were significant (α < 0.05) are highlighted in **bold**. * Variables that were not significant, but remained in the final model. Nest of origin was not significant in any of the models.
<table>
<thead>
<tr>
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<th>Treatment X Age at capture</th>
<th>Age at capture</th>
<th>Treatment X Age at capture</th>
<th>HVC volume</th>
</tr>
</thead>
<tbody>
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<td><strong>Telencephalon</strong></td>
<td></td>
<td>8.680</td>
<td>1, 26</td>
<td>0.007</td>
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<tr>
<td>Treatment</td>
<td>0.102</td>
<td>1, 28</td>
<td>0.752</td>
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</tr>
<tr>
<td><strong>HVC-to-RA % area of MBP</strong></td>
<td></td>
<td>5.927</td>
<td>1, 25</td>
<td>0.022</td>
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<td>Age at capture</td>
<td>0.811</td>
<td>1, 28</td>
<td>0.376</td>
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</tr>
<tr>
<td>Treatment X Age at capture</td>
<td>1.455</td>
<td>1, 28</td>
<td>0.238</td>
<td></td>
</tr>
<tr>
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<td>8.498</td>
<td>1, 25</td>
<td>0.007</td>
</tr>
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<td>Treatment</td>
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<td>1, 28</td>
<td>0.744</td>
<td></td>
</tr>
<tr>
<td><strong>HVC-to-RA average size of tract</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age at capture</td>
<td>0.811</td>
<td>1, 28</td>
<td>0.376</td>
<td></td>
</tr>
<tr>
<td>Treatment X Age at capture</td>
<td>1.455</td>
<td>1, 28</td>
<td>0.238</td>
<td></td>
</tr>
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<td><strong>HVC volume</strong></td>
<td></td>
<td>2.911</td>
<td>1, 28</td>
<td>0.099</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.473</td>
<td>1, 28</td>
<td>0.497</td>
<td></td>
</tr>
<tr>
<td><strong>HVC-to-RA no. of stained sections</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at capture</td>
<td>2.110</td>
<td>1, 28</td>
<td>0.157</td>
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<td>Treatment X Age at capture</td>
<td>0.243</td>
<td>1, 28</td>
<td>0.626</td>
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<td><strong>HVC volume</strong></td>
<td></td>
<td>2.213</td>
<td>1, 28</td>
<td>0.626</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.473</td>
<td>1, 28</td>
<td>0.497</td>
<td></td>
</tr>
<tr>
<td><strong>LMV % area of MBP</strong></td>
<td></td>
<td>0.813</td>
<td>1, 28</td>
<td>0.375</td>
</tr>
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<td>Age at capture</td>
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<td>1, 28</td>
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<td>Treatment X Age at capture</td>
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<td>1, 28</td>
<td>0.147</td>
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<td></td>
<td>1.126</td>
<td>1, 28</td>
<td>0.298</td>
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<tr>
<td>Treatment</td>
<td>1.593</td>
<td>1, 28</td>
<td>0.217</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Age at capture</td>
<td>2.171</td>
<td>1, 28</td>
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<tr>
<td>Treatment X Age at capture</td>
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<td>1, 28</td>
<td>0.097</td>
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<tr>
<td><strong>HVC volume</strong></td>
<td></td>
<td>0.281</td>
<td>1, 28</td>
<td>0.600</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.116</td>
<td>1, 28</td>
<td>0.736</td>
<td></td>
</tr>
<tr>
<td><strong>LMV no. of stained sections</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at capture</td>
<td>0.061</td>
<td>1, 28</td>
<td>0.806</td>
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</tr>
<tr>
<td>Treatment X Age at capture</td>
<td>1.807</td>
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</tr>
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<td><strong>HVC volume</strong></td>
<td></td>
<td>0.015</td>
<td>1, 28</td>
<td>0.904</td>
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</tbody>
</table>

Covariates are in *italics.* Variables that were significant (*∝ < 0.05*) are highlighted in **bold.**
5.3.3 Myelination of the HVC-to-RA tract and LMV tract

5.3.3.1 All ages

In general, all birds increased myelination of the HVC-to-RA tract with age (% Area: \( F_{2,56} = 14.937, P < 0.00001 \); Average Fiber Size: \( F_{2,56} = 13.732, P < 0.0001 \); Number of Fibers: \( F_{2,60} = 7.186, P < 0.01 \); Figure 5-4b; Figure 5-6). Juvenile birds had significantly less myelin immunoreactivity, fewer fibers, and each individual fiber was significantly smaller than those in yearlings (post-hoc tests: all Ps < 0.001) and adults (post-hoc tests: all Ps < 0.001; Figure 5-4b;). Compared to adults, yearlings had a comparable amount of % area of myelin (\( P = 0.552 \)) and fibers (\( P = 0.608 \)), but the average size of their fibers was still smaller than in adult birds (post-hoc test \( P = 0.047 \); Figure 4b;). HVC size was a significant positive covariate for total area of myelin detected (HVC: \( F_{1,56} = 8.313, P < 0.01 \)) and average fiber size (HVC: \( F_{1,56} = 6.807 \),

![Photomicrographs of MBP-stained sagittal sections of the HVC-to-RA tract](image)

Figure 5-6 Photomicrographs of MBP-stained sagittal sections of the HVC-to-RA tract for representative (a) control juvenile, (b) yearling, and (b) adult males. Ventral is down and caudal is right in all photos. Scale bar = 500 µm
$P = 0.012$), but not for the number of fibers (HVC: $F_{1,60} = 0.057, P = 0.813$). Thus, birds with larger HVCs have more myelin, larger fibers, but not necessarily more fibers in the HVC to RA tract.

Myelination of the of LMV tract also increased with age ($F_{2,57} = 4.340, P = 0.018$; Figure 5-4c; Figure 5-7). However, this effect was due to juvenile birds having a smaller

Figure 5-7 Photomicrographs of MBP-stained sagittal sections of the LMV tract for representative control (a) juvenile, (b) yearling, and (c) adult males. Ventral is down and caudal is right in all photos. Scale bar = 500 µm
% area of myelin than yearlings \((P = 0.041)\) and adults \((P = 0.027; \text{ Figure 5-4c})\); there was no difference between yearling and adult birds \((P = 1.000)\). However, average myelin fiber size did not change across ages \((F_{2,60} = 0.461, P = 0.633)\) and there was no difference in the number of individual fibers labeled \((F_{2,60} = 0.693, P = 0.504; \text{ Figure 5-4c})\). HVC size was not a significant covariate for any of the measures of the LMV tract \((% \text{ Area}: F_{1,60} = 0.854, P = 0.395; \text{ Average Fiber Size}: F_{1,60} = 0.053, P = 0.819; \text{ Number of Fibers}: F_{1,60} = 0.106, P = 0.746)\).

### 5.3.3.2 Juveniles

Being raised in the treatment group did not significantly affect the amount of MBP immunoreactivity, average fiber size, or the number of fibers for either the HVC-to-RA tract or the LMV tract \((\text{ Figure 5-8a}; \text{ Table 2})\). While not significant, HVC remained in the final model for % area of myelin immunoreactivity of the HVC-to-RA tract, as birds with larger HVCs tended to have more myelin overall. HVC size was not a significant covariate in any of the remaining measures of the HVC-to-RA tract or any of the LMV tract measures.

### 5.3.3.3 Yearlings

Being raised in the treatment group did not alter the % area of myelin immunoreactivity of the HVC-to-RA tract or the number of fibers \((\text{ Figure 5-8b}; \text{ Table 3})\). While not significant, developmental treatment did remain in the final model for average fiber size. Treatment birds had larger fibers on average than control birds \((\text{ Figure 5-8b})\). Developmental treatment did not affect any of the measures of the LMV tract \((\text{ Table 3})\).

Participation in the cognitive treatment affected myelination of the HVC-to-RA tract, but not the LMV tract \((\text{ Table 3})\). Birds that participated had a smaller % area of
Figure 5-8 Effects of the developmental treatment (Control: white bars; Treatment: black bars) on myelination of the HVC-to-RA tract across (a) juvenile, (b) yearling, and (c) adult starlings. (A) Myelination in juvenile treatment and control males is comparable. (B) Yearlings from both groups have comparable proportions of myelin (left y-axis), but treatment birds on average have larger myelinated fibers than controls (right y-axis). (C) In adulthood, there was no difference in myelination across treatment and control birds. + P < 0.06
myelin and smaller average fiber size of the HVC-to-RA tract (Table 3). For the HVC-to-RA tract and the LMV tract, none of the interactions between developmental treatment and cognitive treatment were significant (Table 3).

HVC size was a significant positive covariate in the % area of myelin immunoreactivity and the average fiber size in the HVC-to-RA tract, but was not a significant covariate for any of the LMV tract measures (Table 3).

### 5.3.3.4 Adults

Being raised in the treatment group did not affect any of the myelination measures for the HVC-to-RA tract or the LMV tract into adulthood (Figure 5-8c; Table 4). The age at which birds were captured did not affect myelination of the LMV tract, but was a significant factor for % area of myelin immunoreactivity of the HVC-to-RA tract. Birds that were caught as nestlings had more myelin that birds that were caught as juveniles (Table 4).

HVC size was a significant positive covariate in the % area of myelin immunoreactivity in the HVC-to-RA tract, but was not a significant covariate in any of the other HVC-to-RA tract measures or any of the LMV measures. None of the interactions between treatment and age at capture were significant for measures of the HVC-to-RA tract or the LMV tract.

### 5.4 Discussion

In an open-ended song learner, the developmental treatment adversely affected song and its supporting neural structures, but the effects diminished with age. This demonstrates that open-ended song learners may recover from the effects of early-life
developmental stressors. In Farrell et al. (2011), starlings reared in the treatment group sang shorter song bouts than control birds during their first breeding season as yearlings, but by their second breeding season (this chapter) treatment birds recovered their song bout length to be equal to their control counterparts. Parallel neural analysis in these adults found no long-term effect of the treatment on song nuclei volumes or myelination within the tracts of the song-control system. Rather, the most substantial neural deficits on song nuclei were observed immediately following the termination of the treatment in juvenile birds (Figure 5-5a). Though some deficits were still detected later in yearlings, no effects of the developmental treatment were visible in the adult 2-year old birds. Conversely, myelination of the song-control system occurred predominantly after the developmental treatment. Thus, the treatment had little effect on myelination in juveniles. However, yearling birds that experienced chronic food-restriction in the months following the developmental treatment, as a result of participating in the cognitive treatment (Chapter 3), showed a notable reduction in the % area and size of myelinated fibers within the HVC-to-RA tract. In general, the age-related variation in myelination of the tracts within song-control system is in accordance with the age-related variation previously documented in song behaviour and song-control nuclei volumes in this species (Bernard et al. 1996). Each phase of song-control system development was affected most by the environmental factors overlapping with it. In conclusion, the effects of developmental stressors in species that are open-ended song learners suggest that song quality is not fixed by early developmental, but rather more proximate, environmental conditions.
5.4.1 Treatment effects on song quality

Early nutritional stressors have negative effects on song bout length and repertoire size in starlings (Buchanan et al. 2003; Spencer et al. 2004; Farrell et al. 2011), yet it appears that these effects are not permanent. Previous work on canaries (Serinus canaria), also open-ended learners, and starlings, has examined how acoustic and social isolation affects song learning across multiple years (Chaiken and Böhner 2007; Leitner and Catchpole 2007). Individuals raised in acoustic or social isolation during the first year of life, if exposed to normal song-learning conditions the following year, can recover many normal song qualities (i.e., appropriate repertoire size and normal syntax; Chaiken and Böhner 2007; Leitner and Catchpole 2007). My adult males in this study were housed in identical conditions (e.g., ad libitum food, exposed to same tutors, etc.) between their first and second breeding seasons. Combined, my findings and previous work suggest that an open-ended learning strategy is adaptable and that song characteristics are largely influenced by proximate conditions at the time of learning and/or production. Although, it is possible that other dimensions of song learning (e.g., repertoire size, syntax), for which my analyses were not sensitive enough to detect, could have been affected permanently.

In contrast to my previous results (Farrell et al. 2011), my experimental treatment did not reduce mean song bout length in the yearling birds assessed herein. This may be for several reasons. Compared to Farrell et al. (2011), my sample size is smaller and most males raised in the control treatment did experience some food intake manipulation as a result of participating in the cognitive experiments in Chapter 3. This factor alone reduced myelination within the song-control system of yearlings (Table 5-3). While an
unintentional factor, song bout length was likely affected adversely in these control males, which given the small sample size, reduced the likelihood of detecting an effect of the developmental treatment. Interestingly, the three yearling males who did not sing in the courtship condition were males that were raised in the treatment group. This suggests that motivation to sing, as well as song complexity, may be affected by developmental stressors.

5.4.2 Treatment effects on nuclei and myelination of the song-control system

While European starling song behaviour has been assessed within the framework of the developmental stress hypothesis, mine is the first study to conduct parallel brain analyses. In starlings, the song-control system and its connections rapidly develop during post-fledgling, through the juvenile period (Casto and Ball 1994), and continue in to adulthood (Bernard et al. 1996). Comparable to my behaviour results, my developmental treatment had no permanent effect on the song-control system in adult starlings (as detected by the sensitivity of the measures used here). Juvenile males showed the most severe reaction to the treatment – they had smaller HVCs and a trend towards smaller RAs than control birds. HVC in particular is highly susceptible to environmental stress during the fledgling stage. My results are similar to studies of fledgling song sparrows (Melospiza melodia: MacDonald et al. 2006) and zebra finches (Taeniopygia guttata: Honarmand et al. 2015) that have noted reductions in HVC volume and rates of neurogenesis in response to early environmental stressors. My results for my yearling birds differ from what has been found to date – only area X volume was reduced in my treatment birds. Unlike studies in other species (Nowicki et al. 2002; Spencer et al. 2003;
Spencer et al. 2005; Schmidt et al. 2013), no deficits were detected in HVC or RA volumes. Area X develops later than HVC and RA (Bottjer et al. 1985), and therefore there may have been less overlap between area X growth and the developmental treatment. Prior to the breeding season, area X increases due to input received through efferent projections from HVC (Brenowitz 2004) and without this input the seasonal increases in area X would not occur (Brenowitz and Lent 2001; Brenowitz and Lent 2002). It is possible, that while HVC volume was unaffected, that treatment males may have reduced projections or weaker transynaptic connectivity from HVC to area X, which could cause a reduction in volume of area X. While I did not observe a difference in myelination of the LMV tract, which includes projections from HVC to area X, I only quantified a portion of the tract, and not the sections where area X was most prominent. In addition to carrying HVC-to-area X projections, the LMV tract also contains projections from other nuclei within the mesopallium and hyperpallium (Jarvis et al. 2013). I was unable to determine in the current experiment the source and targets of the axonal projections that I measured. Future studies could examine the link between differences in area X with markers of myelination to test this hypothesis.

When the developmental treatment ended when birds were 4 months of age, myelination within the song-control system was in its early stages. Subsequently, myelination was largely affected by environmental factors post-treatment. Males that participated in the cognitive experiments (Chapter 3) had less myelin and smaller sized fibers within the HVC-to-RA tract compared to males who did not participate. The HVC-to-RA tract comprises part of the motor pathway of the song-control system, which is essential for facilitating the complex motor movement necessary for song production.
Increased myelination is associated with enhanced behavioural movement, likely due to increased conduction velocity that facilitates the synchronization of complex motor patterns (Alix and Domingues 2011). Despite less myelin between males based upon participation in the cognitive experiments, I did not observe a difference between them in song bout length. However, other measures of song quality that better assess motor performance (e.g., syllable stereotypy) may have differed between treatment groups. HVC volume correlated with myelination of the HVC-to-RA tract, but it did not correlate for myelin measures of the LMV tract. However, there were no differences in myelination within the LMV tract. HVC volume may not have correlated with LMV tract myelination because projections of the LMV tract analyzed may have contained projections from other nuclei within the mesopallium and hyperpallium (Jarvis et al. 2013).

Slowed growth observed in the song-control system could be explained by several non-mutually exclusive factors (Spencer and MacDougall-Shackleton 2011). First, my food manipulations may have elevated plasma glucocorticoid levels via changes to the hypothalamic-pituitary-adrenal (HPA) axis. Although I did not quantify glucocorticoid levels, others who have manipulated food quality (Kitaysky et al. 2001), caloric restriction (Kempster et al. 2007), and food predictability (Buchanan et al. 2003) have found that such manipulations can increase either baseline or stress-induced levels of glucocorticoids. Second, food unpredictability may have altered how birds allocated resources to the development of various physiological systems. In Chapter 2 (Farrell et al. 2015a), I noted that treatment birds had less body fat during the treatment period, despite weighing the same as control birds. Less body fat is indicative that treatment birds were
funneling resources into somatic growth and maintenance to keep pace with controls, and therefore had fewer resources to store as fat for later use. Smaller HVC and RA volumes in juvenile treatment birds suggest that development of the song-control system was superseded by more critical systems. Similarly, yearlings that were food-restricted as a function of the experiments in Chapter 3 had reduced myelination of the HVC-to-RA tract. Again, food-restriction likely caused these males to allocate resources towards more critical systems than the song-control system. Furthermore, once the developmental treatment ended, treatment birds accelerated growth significantly. An accelerated growth strategy suggests birds slowed growth of non-critical systems during a period of time when resources were limited (Schew and Ricklefs 1998). This accelerated growth following the treatment may have aided HVC and RA volumes to recover to the levels of controls by the yearling phase. Although I found no long-term deficits on song and the song-control system, accelerated growth can come at the expense of other physiological systems (Metcalf and Monaghan 2001). It is possible that other systems were adversely affected and/or the effects may manifest even later in life (Metcalf and Monaghan 2001). Lastly, treatment birds, and birds in the experiment in Chapter 3, may have altered their behaviour to conserve energy and make better use of their limited resources. Behavioural changes, such as sleeping or foraging for food, could aid with a resources shortage but would divert attention away from listening to tutor songs and/or producing song. Such changes could have contributed to smaller song-control nuclei, a reduction in song production, which could contribute to a reduction of myelination in the HVC-to-RA tract.
5.4.3 Age related variation in the song-control system

The temporary adverse effects of the experimental treatment did not inhibit the age-related increases in song and song-control system development that is typically observed within this species (Eens et al. 1992; Bernard et al. 1996). My results are in line with previous studies that show an approximate song bout length increase of approximately 7 s from yearling to adult song. Previous studies found that only HVC and RA increased significantly from yearling to adult (2 years or more), while there was a trend for area X to get larger (Bernard et al. 1996). Here, all nuclei, including area X, increased significantly in volume across each stage of development to the next. Fewer birds were analyzed in Bernard et al. (1996) and my larger sample size may have aided in detecting the age effect in area X.

Age-related variation in myelination of the song-control system was prominent. Myelination of the song-controls system increased most notably between the juvenile to yearling phase, with both the HVC-to-RA and LMV tracts increasing across all myelin measures. From yearling to adult, only the HVC-to-RA tract showed age related growth with an increase in average fiber size. Myelination is activity driven (Alix and Domingues 2011). Myelination may increase with age because song bout length and/or total song production increases with age. Comparatively, the increased myelination could increase singing behaviour and contribute to the age-related increases in song bout length. Myelination may be driven by output from HVC, and here I found that measures of myelination of the HVC-to-RA tract positively correlated with HVC volume. It is difficult to assess if behaviour drives neural development, or vice versa (Brenowitz 2004). Human and non-human animal studies have shown that individuals who interact
more with certain environmental stimuli, or engage repetitively in certain motor
movements, can experience an increase in white matter plasticity (Gyllensten et al. 1967;
Bengtsson et al. 2005; Scholz et al. 2009). As increased myelination is associated with
enhanced behavioural movement (Alix and Domingues 2011), more myelin in adult birds
along the HVC-to-RA tract likely facilitates the complex motor movement necessary for
song production. The LMV tract comprises the anterior forebrain pathway of the song-
control system, which is responsible for song learning and not for coordinating motor
movements. This may explain why the LMV tract does not experience a notable increase
in myelination from the yearling to adult phase.

5.4.4 Developmental Stress Hypothesis: from closed to open-ended
learners

A comparative approach may aid in understanding how developmental stress can
affect different aspects of the song-control system, song behaviour, and ultimately
reproductive fitness. Song learning varies along a continuum of closed- to open-ended
learning strategies (Brenowitz and Beecher 2005), yet seasonal changes in the song-
control system appear to be nearly universal for non-tropical birds (Ball et al. 2004).
Open-ended song learning may have evolved out of seasonal plasticity, potentially as a
pre-adaptation to conserve energy requirements during the non-breeding season
(Brenowitz 2004). Starlings may revert to a ‘juvenile pattern of synaptic connectivity’
(Brenowitz and Beecher 2005), which may buffer the song-control system from
permanent damage caused by early-life stressors. Often there are costs associated with
‘catch-up growth’ (Metcalfe and Monaghan 2001), and thus it is unknown if there were
any adverse consequences in the starlings that did so.
Even in closed-ended learners, seasonal plasticity may still allow for the song-control system and aspects of song to recover from developmental stressors. Song-sparrows, a closed ended learner, experience seasonal changes in volumes and rates of neurogenesis, which coincide with changes in song stereotypy throughout the seasons (Smith et al. 1997; Tramontin and Brenowitz 1999). Song sparrows that were food-restricted had smaller HVCs when measured at 3 weeks of age (MacDonald et al. 2006), although there was no long-term effect on HVC volume or neuron number when assessed in adulthood (Schmidt et al. 2013). Songs from these developmentally stressed males were largely affected only in measures of song learning (e.g., smaller repertoire size, poorer copying of tutor song), but measures of song performance (e.g., song stereotypy) were unaffected. Developmental stress may permanently affect aspects of song that crystallize while the developmental stressor is ongoing, yet seasonal plasticity may allow even closed-ended learners to catch-up certain aspects of song that were developmentally delayed due to slowed growth induced by a poor early-life environments. In adult song sparrows, elevated corticosterone levels are known to reduce neurogenesis and HVC volume (Newman et al. 2010), and therefore a stressful winter may be signaled through a reduction in song performance the following spring. A male’s song may be indicative of his ability to cope with developmental and adult stressors (song learning versus performance characteristics, respectively), thus signaling multiple facets of a male’s quality (MacDougall-Shackleton and Spencer 2012).

Studying the effects of developmental stress on species that vary along the closed- and open-ended learning continuum may give insight into the evolution of neural mechanisms between closed- and open-ended learners. For example, brown-headed
cowbirds (*Molothrus ater*) delay song memorization until their second year of life and song production until their third (O’Loghlen and Rothstein 2002). In this species, there are potentially multiple windows where stressors could affect song-learning processes, resulting in multiple possible outcomes on song production, and ultimately fitness.

### 5.4.5 Conclusions

My results suggest that early developmental stress does not have lasting effects on song-learning processes in an open-ended song learner. Development of the song-control system slows when resources are limited, as the development of other critical systems take priority. However, when resources are more plentiful than an accelerated growth strategy is employed to mitigate the adverse effects of a poor early rearing environment. All song-control nuclei were affected at various points in development, along with certain aspects of myelination of the HVC-to-RA tract, which is best explained by taking into account the developmental schedule of the song-control system and if these points in development overlapped with the exposure to developmental stressors. In adulthood, the ability to recover long-term from developmental stress can likely be attributed to changes in neuronal connectivity and plasticity as a function borne out of seasonal plasticity. Using species that vary along the closed- to open-ended song learning continuum will broaden the scope of the developmental stress hypothesis and contribute to the understanding of how developmental stress and adult stress can vary facets of song learning and production. Additional measures of quality, such as immune function or dominance, may fluctuate across the lifespan and song quality from season to season may be indicative of an individual’s current condition.
5.5 References


Chapter 6

6 General Discussion

6.1 Summary

In this thesis, I tested several outstanding issues within the theoretical framework of the *developmental stress hypothesis*. In Chapter 2, I validated that my developmental manipulation induced physiological effects that are characteristic of developing in a stressful early-life environment. I found that birds reared in the treatment group allocated fewer resources towards fat tissue, but gained a significant amount of lean mass in the weeks following the treatment, which is consistent with birds mitigating the effects of poor rearing conditions by differentially allocating resources and exhibiting compensatory growth strategies. In Chapters 2 and 3, I evaluated the long-term effects of the developmental treatment on physiological and cognitive traits associated with phenotypic quality in adulthood. In Chapter 2, I found that treatment males reduced baseline androgen levels in adulthood, but females were unaffected. However, accelerated growth following the treatment period in females was negatively related to total androgen secretion in adulthood during a gonadotropin-releasing hormone (GnRH) challenge. In Chapter 3, I found the developmental treatment slowed the acquisition of learning on auditory tasks assessing absolute and relative frequency discriminations, but in females only. However, both males and females reared in the treatment group showed deficits on a colour association task. Although the developmental treatment induced cognitive deficits, I did not find any support that performance on different tasks were correlated to each other. A lack of such an association indicates that the cognitive
abilities necessary for auditory learning do not reflect cognitive performance in other domains.

In Chapters 4 and 5 I assessed how the developmental treatment affected behaviour in mating contexts and brain development. In Chapter 4, I found that the developmental treatment reduced females’ preference for conspecific song and compared to controls these females had a reduced neural response in auditory forebrain regions when listening to playbacks of conspecific song. Lastly, in Chapter 5, I assessed the effects of the developmental treatment on song production and the song-control system at several developmental milestones in an open-ended song learning species. Developmental stress did reduce the volumes of song nuclei HVC and RA in juvenile males, but by the first breeding season these nuclei were comparable in size to control males. In the first breeding season, area X volume was reduced by the developmental treatment, and the food-restriction in the juvenile period that occurred as function of the cognitive tests completed in Chapter 3 was found to reduce myelination of the HVC-to-RA tract involved in song production. Yet, no long-term effects of the developmental treatment were detected in the second breeding season on song bout length (a measure of song quality in starlings), song-control system volumes, or myelination of tracts within the song-control system. Therefore, adverse effects of developmental stress were noted, but diminished with age in this open-ended song learner.

My thesis is a comprehensive assessment of the developmental stress hypothesis and is novel in several respects. First, differential resource allocation is a mechanism by which developmental stress is suspected to exert its effects. By using quantitative magnetic resonance (QMR) I was able to capture this phenomenon by detecting changes
in body composition between lean and fat tissue during, and following, the developmental manipulation. Second, this is the first study to provide an assessment of how auditory capabilities of both sexes are affected by developmental stress. Third, this is the first study of the brains of European starlings within the framework of the developmental stress hypothesis. And fourth, this study is the first to adopt a comprehensive analysis of how developmental stress affects behaviour and the brain in an open-ended song learning species through various developmental milestones. Furthermore, these effects quantified changes in myelination within the tracts of the song-control system, which is a relatively understudied neural measure and entirely novel within the developmental stress hypothesis literature.

6.2 Sex-specific effects of developmental stress

The studies presented herein demonstrate that early-life stress has sex-specific effects. Females exhibited greater deficits on auditory learning tasks, and reduced neuronal activation in auditory forebrain regions when listening to conspecific song. In contrast, males did not exhibit any auditory learning deficits, and there were no long-term deficits observed in the size or myelination of the song-control system. However, HPG function was affected in males in adulthood. Both sexes exhibited accelerated growth following termination of the developmental treatment. Accelerated, or compensatory, growth is known to be costly and associated with reduced lifespan (Metcalf and Monaghan 2001), but can lead to beneficial increases in short-term reproductive success (Metcalf and Monaghan 2003). Each sex should strategically allocate resources to the systems that have the largest influence on fitness (Metcalf and Monaghan 2001). In general, increases in body mass for developing females is driven by selection on
fecundity, while increases in males is associated with sexually selected traits (Andersson 1994; Blanckenhorn 2000). To maximize current reproductive opportunities females should direct resources from accelerated growth towards measures of fecundity, but males should direct resources to sexually selected traits.

In songbirds, males will incur a larger reproductive benefit from developing the auditory abilities and neural structures for auditory and song functions compared to females. Compromised functioning in the male song-control system would have substantial impacts on future mating success (i.e., it is better to sing poorly than not at all). When raised on a poor quality diet, male zebra finches chose to accelerate the growth and development of sexually selected traits to maximize current reproduction at the cost of future reproduction (Birkhead et al. 1999). As adults, these males scored similarly to control males on the primary sexual trait of sperm viability, and the secondary measures of beak colouration and song rate; however, these males had shorter lifespans than controls (Birkhead et al. 1999). I did not detect any long-term effects of developmental stress on auditory learning and song-control system development in males, but if I had examined males past the second breeding season I may have detected that the costs of accelerating song development were being paid through reduced lifespan, and/or a faster decline in song abilities in adulthood. Accelerated growth is costly, therefore considering the time scale when its associated costs are paid is a central tenet of life history theory (Wingfield et al. 1998; Metcalfe and Monaghan 2001). Future studies of developmental stress should consider incorporating measures that could assess how costs are paid over a longer time-scale. Lifespan is laborious to measure in long-lived species, but measures that are reflective of the aging process, such as measures of
oxidative stress (Alonso-Alvarez et al. 2007) and telomere attrition (Bekaert et al. 2005; Nettle et al. 2015), could be used in its place.

Females should be concerned with maintaining body condition, and at its expense trade-off developing the neural circuitry necessary for discriminating complex auditory signals. As there is a male-bias in the adult starling sex ratio (Mayr 1939; Kessel 1957), a yearling female is more likely to reproduce than a yearling male (Flux and Flux 1982). Therefore, a female may net more fitness benefits by maintaining good body condition for reproduction, rather than develop the ability to differentiate nuances in the quality of male song (i.e., it is better to have the resources for reproduction than the ability to identify the best singer). Data from zebra finches and great tits support a positive association between female juvenile body condition and future reproductive success: juvenile body mass in females was predictive of the size of future clutch sizes (Haywood and Perrins 1992). Females developing in stressful environments may alter their physiology to maximize short-term reproductive strategies. I found that females who accelerated growth the most following the developmental treatment were females who had the lowest integrated androgen levels. Higher levels of androgens in female songbirds are associated with delayed onset of reproduction (Clotfelter et al. 2004), reduced breeding success (López-Rull and Gil 2009), and reduced fecundity (Rutkowska et al. 2005). Therefore, females could mediate life-history trade-offs through androgen regulation by lowering androgen production to maximize current reproductive success (Veiga and Polo 2008; Cain and Ketterson 2013).
6.3 Developmental timing of exposure to environmental stress

In designing the auditory experiments in Chapter 3, I did not predict that the weight-restriction protocol used to regulate motivation would induce as large, if not larger, effects on various physiological, behavioural, and neural measures compared to the earlier developmental treatment. Controlling for participating in the cognitive experiments explained a significant amount of variation in neural and behavioural measurements in females and, to a lesser extent, males. Most research focuses on the prenatal and postnatal developmental period, but the adolescent period preceding the first breeding season is still one where behaviours and the brain are plastic and influenced by environmental factors that can shape the adult phenotype (Lupien et al. 2009).

In females, participation in the cognitive experiments adversely affected behavioural and neural measures – preference for conspecific song was weakened, and expression of the immediate-early gene Zenk was reduced in auditory forebrain regions. And although the volumes of all three song-control nuclei (HVC, RA, and area X) were not affected by participation in the auditory experiments, it was the final factor to be removed from the statistical model. On average, nuclei volumes were smaller in females who participated in the cognitive experiments relative to females who did not (see Figure 4-6b). Female brains were collected approximately 6 months after the cognitive treatment was terminated; therefore, the nuclei volumes had time to recuperate from any initial volume loss. The ability of song-control nuclei to recuperate from chronic food-restriction following restoration to an ad libitum diet was noted across the juvenile and yearling male brains analyzed in Chapter 5. Similarly, young song-sparrows that experience food-restriction had smaller HVCs, but by adulthood there was no longer any
volume differences across males reared in stressful and control developmental conditions (MacDonald et al. 2006; Schmidt et al. 2013). The ability of the song-control nuclei to recover, at least in size, may be possible due to alterations in neural plasticity borne out of seasonal plasticity (Brenowitz 2004).

Participating in the cognitive experiments also altered female HPG function, resulting in higher baseline plasma androgen levels. Experiencing chronic food-restriction in the months preceding the breeding season could have adversely affected a female’s quality. Elevated androgens are negatively associated with measures of fecundity (Clotfelter et al. 2004; Rutkowska et al. 2005; López-Rull and Gil 2009), and weaker female mate preferences (McGlothlin et al. 2004). Female preferences, if condition-dependent, could be indicative of a female’s quality (Cotton et al. 2006; Buchanan et al. 2013). If participation in the cognitive experiments reduced female condition, mediated through androgen regulation, I would predict an association between androgen levels and the strength of females’ song preferences. I assessed this prediction by testing the relationship between both measures of song preference calculated in Chapter 4 and the three measures of HPG function calculated in Chapter 2 (Table 6-1). As predicted, the relationship between baseline androgen levels and preference for conspecific song was significantly negative: females with higher baseline androgen levels showed weaker preference towards conspecific song.

For purposes of reproduction, higher androgen levels in poor quality females may mediate a suite of behaviours to aid them compete against females in better condition. First, if androgens weaken choosiness for a male trait, this results in a female selecting a
Table 6-1 Spearman correlation matrix assessing the relationship between song preferences and HPG function. Preference ratios calculated in both preference tests in Chapter 4 (conspecific vs. heterospecific song; long vs. short starling song bouts) were compared to the three measures of HPG function calculated in Chapter 2 (Baseline: baseline androgen levels; Peak: peak androgen levels induced through a gonadotropin-releasing hormone [GnRH] challenge; Integrated: total androgen secretion calculated through the duration of the GnRH challenge).

<table>
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<tr>
<th>HPG Measures</th>
<th>Preference for Conspecific vs. Heterospecific Song</th>
<th>Preference for Long vs. Short starling song bouts</th>
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<tr>
<td>Baseline</td>
<td></td>
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<tr>
<td>$r_s$</td>
<td>-0.610</td>
<td>0.121</td>
</tr>
<tr>
<td>$P$</td>
<td>0.004</td>
<td>0.622</td>
</tr>
<tr>
<td>$N$</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Peak</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r_s$</td>
<td>0.005</td>
<td>-0.032</td>
</tr>
<tr>
<td>$P$</td>
<td>0.985</td>
<td>0.898</td>
</tr>
<tr>
<td>$N$</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Integrated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r_s$</td>
<td>-0.438</td>
<td>0.065</td>
</tr>
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<td>0.054</td>
<td>0.792</td>
</tr>
<tr>
<td>$N$</td>
<td>20</td>
<td>19</td>
</tr>
</tbody>
</table>

The Bonferroni correction ($\alpha$ level of significance ÷ number of contrasts) was applied to control for type I error making multiple comparisons. **Bolded values** denote comparisons that were significant after applying the Bonferroni correction ($0.05/6 = 0.008$).

mate in less time and less energy is expended by not sampling several males (Dale et al. 1990; Cotton et al. 2006). Secondly, increased androgens will aid in intrasexual competition. In female starlings, increased testosterone levels in the breeding season are associated with higher levels of intrasexual aggression (Sandell 2007), which can help to ensure a monogamous pair bond and prevent a male partner from giving resources to another female (Sandell 1998). In the congeneric spotless starling (*Sturnus unicolor*), increased testosterone enhanced a female’s ability to acquire and maintain a breeding site (Veiga and Polo 2008). While increasing androgens reduces fecundity, a poor quality female may be attempting to maximize current reproduction by employing a ‘live fast, die young’ strategy that has her trying to ‘make the best of a bad situation’ given her
limited resources (Metcalf and Monaghan 2001; MacDougall-Shackleton and Spencer 2012).

Most of the males reared in the developmental control condition also participated in the cognitive experiment. Therefore, almost all males in these experiments experienced some chronic food-restriction prior to their first breeding season. While participation in the cognitive testing did not affect song-control system nuclei volumes, it did reduce the average myelinated fiber tract size in the HVC-to-RA tract. The HVC-to-RA tract controls the motor production of song (Nottebohm et al. 1976; Simpson and Vicario 1990), and less myelin within this tract could affect song production and potentially shorten mean song bout length, or reduce total amount of time spent singing (i.e., song rate). Participation by males in the cognitive experiments may have compromised my ability to detect an effect of the early developmental treatment on male song quality.

Compared to a previous study I conducted where I did find a treatment effect on mean song bout length in birds raised in the 2009 cohort (Farrell 2011), the average mean song bout length in the birds reared in the 2012 cohort is longer, there is less variation in mean song bout length, and the sample sizes are smaller, which are all factors that make it difficult to detect a small effect size (Figure 6-1).

The effects of participation in the cognitive experiments highlights that the song-control system in this open-ended song learner is plastic well into the months preceding the breeding season. This result indicates a potential problem of weight-restriction practices that are commonly employed in cognitive testing with animals (Toth and Gardiner 2000). Extended periods of weight-restriction could affect brain development and aspects of physiology, which may compromise behavioural results (Heiderstadt et al.
Moreover, weight-restriction enforced in a period of developmental plasticity may have long-term effects on subsequent measures and/or may alter the strategies that an animal uses to solve a cognitive task. If I were to conduct a similar set of studies again, I would consider altering experimental test procedures to minimize inducing additional physiological stress.

6.4 Evolution of song and song preferences

The developmental stress hypothesis has sparked an array of studies assessing how variation in a sexually selected trait, song, can serve as an honest indicator of a male’s quality. A critical assumption of indicator models is that a sexually selected trait, and preference for it, is heritable (Fisher 1930; Zahavi 1975). Males that possess the trait are honestly advertising their genetic quality and females with strong preferences are advantaged in the sense that they can accurately assess male quality through their
superior trait discrimination. I did not assess heritability directly, but by controlling for the nest birds originated from I found results that support the notion that traits necessary for song learning, and for the discrimination of a biologically relevant signal such as song, have a genetic basis. Nest of origin would account for genetic contributions, as birds from within a nest are likely siblings as the rate intraspecific brood parasitism in starlings is low (less than 1%; Pinxten et al. 1993). However, nest of origin could also explain nongenetic effects, such as parental behaviour prior to capture or variation in hormones deposited into eggs. At the moment, there is some research on the heritability of song, but little on the heritability of song preferences.

Performance in the auditory learning experiments was related to nest of origin, which suggests that there is a genetic component to auditory learning. Auditory learning is a necessary cognitive ability for males to learn songs, and females to discriminate between sexually relevant biological signals (Woolley and Moore 2011). Heritability estimates are low for the neuroanatomy of the song-control system (Woodgate et al. 2014). The nucleus RA appears to be moderately heritable, but HVC size seems almost entirely dependent on environmental factors (Woodgate et al. 2014), despite previous results that suggested otherwise (Airey et al. 2000). However, gene by environment interactions are important for song-control system development and is one possible mechanism by which additive genetic variance is preserved (Woodgate et al. 2014). Female preferences may select for genes that support effective song learning, rather than changes to the neuroanatomical size of song nuclei. Similarly in song sparrows, there may be a heritable component to song performance: sons scored higher on a measure of song performance (trill deviation) if their father’s had larger repertoires, despite not being
raised or tutored by their fathers (Ballentine 2004; Schmidt et al. 2013). While I did not find that nest of origin explained any of the song or song-control system measures analyzed here, genes regulating auditory learning may overlap with song learning ability.

Studies with female insects and fish, using selection lines and quantitative genetics, have found that variation in preferences can be linked to genetic variation (Wilkinson and Reillo 1994; Brooks and Couldridge 1999). However, most studies assessing the heritability of female preferences have found the heritability estimates for a particular male phenotype are low (Brooks and Endler 2001; Schielzeth et al. 2009). Rather, moderate heritability estimates are noted for a female’s responsiveness towards a male trait (Brooks and Endler 2001; Schielzeth et al. 2009). Similarly, I found that while nest of origin was not related to females’ song preferences, it was related to overall levels of neuronal activity in auditory forebrain areas when listening to song. Induction of neuronal activity suggests that females with particular genotypes may have greater activity levels (in part induced by greater neural activity) to biologically relevant stimuli, which may affect a female’s mating behaviour.

6.5 General Conclusions

The developmental stress hypothesis is not simply about explaining how developmental conditions can affect the quality of male song. Rather, the central tenets can be applied to females and other aspects of an individual’s physiological, and cognitive-behavioural phenotype. The studies conducted here used integrative approaches to assess the effects of developmental stress on the physiology, behaviour, and the brain in European starlings. I have made significant contributions to the field by (1) assessing differential resource allocation as a mechanism of developmental stress through the use
of body composition, (2) providing a comprehensive assessment of the effects of developmental stress on female behaviour and neural functioning, and (3) examining how developmental stress affects behaviour and brain development in an open-ended song learning species. My results suggest that developmental stress can induce sex-specific cognitive-behavioural effects, and that developmental stress may not exert as strong a selection pressure on species with an open-ended song learning pattern due to their altered neural plasticity.
6.6 References


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Curriculum Vitae

Tara Marie Farrell

Education

**Ph.D., Psychology**, University of Western Ontario (2010 – 2015)
- Current thesis: *The effects of developmental stress on auditory learning, vocal learning and song preferences in the European starling (Sturnus vulgaris)* with Dr. Scott MacDougall-Shackleton

**M.Sc., Psychology**, University of Western Ontario (2008 – 2010)
- Thesis: *The effects of developmental stress on song-system functioning, cognition and mate choice in the European starling (Sturnus vulgaris)* with Dr. Scott MacDougall-Shackleton

**B.Sc., Psychology (Honors)**, University of Alberta (2003 – 2007)
- Thesis: *Micro-acoustic mechanisms of species discrimination in black-capped (Poecile atricapillus) and mountain (P. gambeli) chickadees* with Dr. Christopher B. Sturdy

Scholarships and Awards

**Scholarships:**

- **PSAC 610 Academic Achievement Scholarship (2013)**
  - Scholarship given to members of PSAC Local 610 at Western on the basis of academic achievement and research excellence
  - Value: ($500)

- **Queen Elizabeth II Graduate Scholarship in Science and Technology (2012-2013)**
  - Value: $10,000

- **NSERC Canadian Graduate Scholarship (2010-2012)**
  - Value: $35,000 annually

- **Ontario Graduate Scholarship (2010-2011)**
  - Value: $15,000 (Declined)

- **NSERC Canadian Graduate Scholarship (2008-2010)**
  - Value: $17,500 (2008-2009)
  - Value: $17,300 (2009-2010)
Western Entrance Scholarship (2008-2009)
  • Value: $8,000

University of Alberta Undergraduate Scholarship (2005, 2006)
  • Value: $1,000 annually

  • Value: $1,000 annually

  • Value: $2,5000

Awards:

Keith Hobson Award (2015)
  • Value: $500

Western Graduate Research Award Fund (2014)
  • Value: $750

Ontario Ecology, Ethology and Evolution Conference - Best Graduate Student Talk Award (2014)
  • Value: $250

Canada-Brazil Award – Joint Research Project (January-February 2012)
  • Stipend given to Canadian members while engaged in research in Brazil ($1400 per month)

Western Graduate Thesis Research Award (2008)
  • Value: $200

Dean’s Silver Medal in Science (2007)
  • Award given to graduating students with superior academic standing enrolled in an Honors program in the Faculty of Science

NSERC Undergraduate Research Award (April-August 2006, April-August 2007)
  • Value: $5,625 each year
Peer-reviewed journal articles


Presentations

**Talks:**


Posters:


*Invited Talks:*


**Teaching Experience**

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**Course instructor** for the following courses at the University of Western Ontario:

- Evolutionary Psychology, Fall 2014 and 2015, Summer 2013, 2014 and 2015
- Hormones & Behaviour, Winter 2014

**Teaching assistant** for the following courses at the University of Western Ontario:

- Introduction to Behavioural and Cognitive Neuroscience, Winter 2014
- Principles of Learning and Behaviour, Winter 2013
- Evolutionary Psychology, Fall 2012 and Fall 2013
- Psychological Aspects of Life-skills, Fall 2011
- Neuroscience of Motivation and Emotion, Fall 2010 and Winter 2011
- Introduction to Test and Measurement 2080, Winter 2010
- Animal Behaviour 3221, Fall 2009
- Introduction to Psychology 1000, Winter 2009
- Educational Psychology 2620, Fall 2008

**Administrative activity and community involvement**

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Western Colloquium Speakers Committee (2014-2015)
- Organized speakers for the Western Psychology colloquium series.
Co-Chair of the Organizing Committee for the 2013 Ontario Ecology, Ethology and Evolution Colloquium, May 2-4, 2013 at Western University (September 2012-August 2013)

- A leadership role in organizing a 125-participants conference, including such duties as venue selection, fundraising efforts, budget planning, contacting plenary speakers and program organization.

Volunteer for Western Neuroscience Summer School at Western University (July 2011)

- Workshop leader for Quantifying Behavioural Data in Captive Songbirds whereby I demonstrated and guided students through the steps involved in capturing, coding and analyzing experimental data.

Graduate Representative of the Work and Resource Planning Committee (2010-2011)

- Represented the interests of graduate students from the Department of Psychology regarding the structuring of courses and hiring of new faculty members.

Psychology Graduate Student Association (2009-2010)

- A leadership role in which the association fosters good working relations between students, faculty and the University of Western Ontario.

Work experience

Research Assistant at the University of Alberta (2005-2008)

- Bioacoustical analysis of bird vocalizations
- Production of experimental acoustic stimuli
- Design and implementation of operant conditioning studies
- Recording of bird song and calls