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Timing of developmental stress and phenotypic plasticity: Effects of nutritional stress at different developmental periods on physiological and cognitive-behavioral traits in the zebra finch (Taeniopygia guttata)

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TIMING OF DEVELOPMENTAL STRESS AND PHENOTYPIC PLASTICITY:
EFFECTS OF NUTRITIONAL STRESS AT DIFFERENT DEVELOPMENTAL
PERIODS ON PHYSIOLOGICAL AND COGNITIVE-BEHAVIORAL TRAITS IN THE
ZEBRA FINCH (*TAENIOPYGIA GUTTATA*)

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by

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Abstract

Developmentally plastic organisms can respond to stressful environmental conditions by altering multiple aspects of their phenotype, often in a permanent fashion. The timing of developmental stress influences these phenotypic alterations because the prioritization of resources to traits necessary to overcome the stressor may be costly for the development of other traits. Despite the importance of this timing, few studies in birds have accounted for it, and those that have usually examined the effect on a single or few variables. This dissertation addresses the outstanding issues regarding i) the effects of timing of developmental stress on developmental plasticity, and ii) the extent to which poor nutritional conditions, as opposed to changes in nutritional conditions, drive phenotypic plasticity. Using zebra finches (*Taeniopygia guttata*) as my study species, I manipulated food accessibility during early and or juvenile development (i.e. before and after nutritional independence) and measured various physiological and cognitive-behavioral traits. Results indicated that timing of stress significantly affected many (but not all) aspects of phenotype measured, including growth rates, body composition, immune function, associative learning, spatial memory, and endocrine function. In particular, nutritional stress during the juvenile period appeared to have strong programming effects on phenotype. Nevertheless, individual differences and sex differences in developmental plasticity greatly moderated the influence that timing of stress had on phenotypic development.

Keywords: avian; nutrition; phenotypic plasticity; phenotypic programming; developmental stress; timing

Co-authorship

Haruka Wada will be a co-author on manuscripts based on data from on chapters 2 and 5 because she assisted with collecting data on immune function (chapter 2) and corticosterone samples (chapter 5). Sean Aitken, Laura Garcia, and Tara Farrell will be co-authors on the manuscript based on data from chapter 3 because they assisted with collecting the data on associative learning, spatial memory, and neophobia. James Brooyman-Quinn will be a co-author on the manuscript based on chapter 4 because he assisted with collecting data on associative learning. Kim Schmidt, Matthew Taves, and Kiran Soma will be co-authors on the manuscript based on data from chapter 5 because they performed the radioimmunoassays on the corticosterone samples. Scott MacDougall-Shackleton will be a co-author on all manuscripts because he was involved in experimental design and manuscript preparation, and provided funding for all the experiments.

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List of Abbreviations

PHD   post-hatch day

H   high feeding treatment

L   low feeding treatment

APC   assay positive control

ABC   assay blank control

IBC   individual blank control

SRBC   sheep red blood cell

BMR   basal metabolic rate

RQ   respiratory quotient

TTC   trials to criterion

HPA   hypothalamic-pituitary-adrenal

CORT   corticosterone

ACTH   adrenocorticotropic hormone

DEX   dexamethasone

CHAPTER 1

GENERAL INTRODUCTION

Both internal and external environments can modify gene expression and consequently influence characteristics of individuals. Environmental effects may also persist long after the change-invoking environmental stimuli have subsided because of enduring changes in gene expression. Environment-induced modifications rarely involve a single, isolated characteristic because development and function of biological systems within an individual are highly interdependent (West-Eberhard 2003). In this thesis I address the consequences of past developmental experiences on various physiological and cognitive-behavioral traits. Specifically, I focus on the importance of the timing of stressful experiences on the development of traits. In this introductory chapter I introduce the concept of phenotypic plasticity, its mechanisms and associated costs, and the idea that developmental environments may have long-lasting *programming* effects on individuals. I then review the literature on the effects of developmental stress (with emphasis on birds) and how responses to developmental stress can be considered a form of phenotypic plasticity. Last I conclude the chapter with a discussion of outstanding research questions regarding the role of timing in moderating the effects of developmental stress and how this thesis addresses these gaps in our knowledge.

1.1 PHENOTYPIC PLASTICITY

The phenotype is defined as "all traits of an organism other than its genome…the individual outside the genome" (West-Eberhard 2003, p.31) and is a product of the complex interplay between the genotype and environment. Phenotypic plasticity is a universal phenomenon among organisms and refers to any type of phenotypic variation in response to environmental signals (Stearns 1989). Such signals include external, internal, biotic, and abiotic factors such as temperature, hormones, parasites, and toxins.

Individuals may alter their morphology, behavior, and life-history tactics in response to environmental factors, and the ways that individuals modify their phenotype will eventually determine their fitness. Not all phenotypic changes are guaranteed to be adaptive or to increase fitness. For example, testosterone enhances expression of sexually selected traits, but may also weaken the immune system (Folstad & Karter 1992). This means that an organism may have to weigh the benefits of enhanced mating success against the cost of

increased susceptibility to parasites and pathogens. Thus, organisms may make trade-offs in their responses to the environment and there may exist alternative strategies for maximizing fitness.

1.1.1 Developmental plasticity

One widespread form of phenotypic plasticity is developmental plasticity. Environmental influences during development can have significant immediate and permanent effects on the phenotype. Temperature dependent sex differentiation in reptiles (Bull 1980), predator-induced change in growth rate and shell morphology of snails (Behrens Yamada et al. 1998; Brönmark et al. 2011), and hormonal and social regulation of caste transition in honey bees (*Apis mellifera;* Huang & Robinson 1992; Sullivan et al. 2000) are just a few examples of developmentally plastic traits. Environmental influences may have varying effects on the phenotype at different ages depending on the developmental schedules of particular traits. For instance, development of the human hippocampus and frontal cortex primarily occurs from birth until 2 years of age, and from approximately 8 until 14 years of age, respectfully (Lupien et al. 2009; Andersen 2003). Traumatic or stressful events during these age ranges greatly increase the risk of developing psychopathologies such as schizophrenia, depression, and post-traumatic stress disorder (PTSD; Andersen & Teicher 2008; Andersen 2003; Heim et al. 2003). Therefore, the heightened sensitivity of developing brain regions to environmental inputs during particular developmental stages means that stress during these times may have exceptionally influential and potentially long-term consequences on the adult phenotype.

The above example suggests that developmental plasticity may be constrained by sensitive periods of development. Sensitive periods, also known as critical periods, are windows of greater neural (as well as behavioral) plasticity; once these windows close, only very minor modifications, if any, can be made to the trait. Many developmental processes have sensitive periods, which explains why many developmental processes are irreversibly plastic. Human speech acquisition and song learning in songbirds are examples of traits that require environmental input and have sensitive periods early in development – the ability to acquire speech or learn species-typical songs is greatly reduced after reaching sexual maturity (Doupe & Kuhl 1999). Because stressful experiences that coincide with sensitive period are

likely to have greater effects on the phenotype, this implies that the timing can be enormously influential in determining the outcomes of developmental stress.

1.1.2 Mechanisms of developmental plasticity

West-Eberhard (2003) describes three mechanisms of developmental plasticity: i) hypervariability and somatic selection, ii) homeostatic mechanisms, and iii) hormones. In hypervariability and somatic selection, random variants, modifications, movements and positions are overproduced, and then some are selectively preserved and others eliminated. For example, operant conditioning involves performance of an assortment of behaviors, of which only some are rewarded and therefore maintained. Brain maturation in humans also undergoes somatic selection, as synaptic pruning of excess neurons and synapses occurs as a result of learning and experience (Huttenlocher 1979).

Homeostatic mechanisms that regulate stability of internal environments can respond flexibly to fluctuations in external environments. Allostasis is the process of maintaining homeostasis through changes in both environmental stimuli and physiological mechanisms (Romero et al. 2009; McEwen & Wingfield 2010). Adjustment of homeostatic mechanisms to unpredictable or uncontrollable environmental factors may be mediated by allostatic regulators. Allostatic load refers to conditions that challenge allostatic mechanisms and make it harder to maintain homeostasis. Subsequently, allostatic overload can occur when the energetic demands of maintaining homeostasis exceed the energy an animal can obtain from its environment. Animals may be able to make behavioral and physiological adjustments to decrease allostatic load, but if these changes are inadequate and allostatic overload persists for too long, then the constant activation of mechanisms designed to reduce allostasis themselves can lead to more enduring phenotypic changes (Romero et al. 2009; McEwen & Wingfield 2010). One type of response to persistent or recurring environmental perturbations that overwhelm allostatic mechanisms is for homeostatic processes to adopt new set points. For example, sensitivity to stress was heightened in adult rodents that experienced brief periods of maternal separation during early postnatal development (Meaney et al. 2001). In short, allostatic regulators are flexible processes that allow animals to maintain homeostasis even in the face of stress. However, prolonged stress may overload allostatic regulators, resulting in potentially permanent phenotypic alterations.

Hormone release is sensitive to environmental influence, allowing organisms to integrate external environmental cues with internal cues, and levels of hormones can directly affect phenotypic traits such as size, color, and activity (Dufty et al. 2002). Hormones can have short-lived activational effects as well as long-lasting organizational effects on phenotype. Control of sexual behavior and development of primary sexual organs are examples of activational and organizational effects of hormones, respectively. Even in low concentrations, presence of particular hormones during development can have significant organizational impact. For example, in mammals, the development of male primary sexual organs is stimulated by the presence of testosterone (Jost et al. 1970).

The activational and organizational effects of hormones are not completely independent, as hormone exposure at one point in time may influence the response or sensitivity to the same hormone at a later time. Temporary elevation of glucocorticoids (a class of steroid hormones released from the adrenals in response to stressful stimuli) is useful because it helps animals to mobilize resources to cope with the stressor, but chronic elevations of glucocorticoids can have long-lasting (and sometimes detrimental) consequences. These long-term effects include modification of sensitivity to glucocorticoids later in life (Meaney et al. 2007).

Long-term programming effects on learning and behavior, homeostatic set points, and endocrine activity resulting from early life experiences are likely attributable to epigenetic changes. Epigenetics refers to mechanisms that program gene expression without changing gene sequence. These mechanisms are initiated in response to environmental influences (McGowan et al. 2008). Alterations to structure of chromatin (i.e. DNA complex and associated histone proteins) through histone modifications affect accessibility of transcription machinery to transcription sites, thereby influencing gene expression. Binding of methyl groups to DNA (DNA methylation) can also cause long-lasting changes in gene expression because enzymes (DNA methyltransferases; DNMTs) and other cellular extracellular signals actively maintain acquired methylation patterns (McGowan et al. 2008). Epigenetic changes may be heritable, but may also be reversed (Szyf 2001 as cited in McGowen et al. 2008) because epigenetic mechanisms are dynamic and responsive to environmental conditions (Goerlich et al. 2012; Naguib & Gil 2005; Anway et al. 2005). Thus, epigenetics describes phenotypic plasticity at the level of genes and may explain how phenotypic variation can occur amongst individuals of the same generation within a population, how plastic responses

can persist throughout an individual's lifetime, and how these changes and adaptations can be passed on to subsequent generations.

To summarize, there are various interacting mechanisms that allow a developing animal to respond flexibly to environmental demands. For instance, sustained glucocorticoid release in response to stress can affect hypervariability and somatic selection in the brain by suppressing neurogenesis and dendritic atrophy (McEwen 2002) and exaggerate reactivity of allostatic mechanisms to future stress (Meaney et al. 2007). These changes may persist due to epigenetic modulation of gene expression (McGowan et al. 2008). I will revisit the importance and role of glucocorticoids in developmental plasticity in more detain in section 1.2.4). In this way, developmental stress produces complex phenotypic changes by affecting multiple physiological and cognitive or behavioral processes at once.

1.1.3 Costs of plasticity

Costs of plasticity refer to any reduction in fitness incurred by plastic individuals relative to non-plastic individuals (Auld et al. 2010). Phenotypic plasticity can be costly if the phenotypic changes are inappropriate or non-adaptive. In addition, plastic individuals incur costs to maintain the ability to detect and respond to environmental cues, and consequently must invest resources in sensory systems and in obtaining information about the environment (DeWitt et al. 1998). The costs of plasticity are revealed when traits developing simultaneously must compete for shared resources, or if the expression of one trait impedes the expression of other traits, such as the antagonistic effects of testosterone on secondary sexual characteristics and immune function (Folstad & Karter 1992). Because these traits are somehow linked (either by the need for the same resources at the same time, or by the process by which one impedes the other), changes in development of one trait is expected to produce changes in the other trait(s). For instance, growth and somatic maintenance are competing processes so organisms face having to make a tradeoff between enhancing growth and extending lifespan (Rollo 2002; Metcalfe 2003).

The example of the tradeoff between growth and longevity illuminates two important points. First, the costs of plasticity may occur on different time scales and thus may not be immediately apparent. Second, the exact costs of plasticity are difficult to calculate because they are so dependent on context. An altered trait may be advantageous in one context, but

disadvantageous in another. In the case of growth and lifespan, trading off longevity for growth might be more beneficial for a short-lived organism than for a longer-lived organism because larger size increases the probability of short-term survival, reproductive success, and fecundity in many animals (Metcalfe 2003). Longer-lived animals may have more opportunities over time to reproduce, so it is probably be less advantageous for them to sacrifice longevity for the sake of growth.

1.2 EFFECTS OF DEVELOPMENTAL STRESS ON PHENOTYPE

The ability of organisms to be developmentally plastic is particularly important when organisms are faced with stress, which are conditions where an environmental demand exceeds the natural regulatory capacity of an organism (Buchanan et al. 2013). Increasingly stressful environments tend to increase phenotypic variation (e.g. Imasheva et al. 1997; Purchase & Moreau 2012). Exposure to stress during development is almost inevitable, and can have profound impacts on phenotype (Lindström et al. 1999; Monaghan 2008). For many animals, developmental stress can include nutritional scarcity, sibling rivalry, predation threats, and extreme weather conditions.

Stress during development is able to alter many aspects of phenotype. Physiological consequences of stress during development include reduced growth rates and body size, sensitization of endocrine systems to stress, and altered brain structure and function (Lupien et al. 2009). In humans, prenatal and perinatal exposure to stress increases risk of cardiovascular and metabolism-related disorders in adulthood (Chrousos 2009). Cognitive and behavioral consequences of stress during development include diminished learning and attentional abilities, and increased anxiety, depression, and fearfulness (Lupien et al. 2009). Furthermore, altered aspects of phenotype as a result of developmental stress may be transmitted to offspring (Naguib & Gil 2005; Naguib et al. 2006).

1.2.1 Glucocorticoids as a mechanism by which environments influence development

Endocrine responses are highly sensitive to environmental conditions and are thus a mechanism by which organisms can detect and respond to their surroundings. In vertebrates, stress activates the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic division of the autonomic nervous systems that release glucocorticoids and catecholamines, respectively, which promote protein catabolism and mobilization of lipid and glucose stores for energy

use. In the brain, glucocorticoids also increase alertness to enhance learning and memory for salient events (reviewed by Meaney 2001; Chrousos 2009). While activation of these stress responses is useful in the short term, chronic elevations of glucocorticoids may be harmful in the long term. In humans, outcomes of long-term chronic stress include increased risk for metabolic disorders, coronary heart disease, depression and anxiety-related disorders, and reduced cognitive function (Chrousos 2009; Pechtel & Pizzaagalli 2011).

The effects of chronically elevated glucocorticoids on health and cognition may be amplified if experienced during development. Being in a state of constant energy mobilization diverts resources away from processes required for growth, maturation, somatic maintenance, reproduction, and immune function (Chrousos 2009). Rapid brain growth and plasticity seen during development may also be hindered by elevated glucocorticoids because they initiate neural atrophy and cell death in regions such as the hippocampus (reviewed by Lupien 2009). In birds, manipulation of the primary glucocorticoid corticosterone can have short- and long-term effects on growth and survival, fecundity, immune function, and behavioral tendencies (Schoech et al. 2011). The effects of developmentally elevated glucocorticoids on a phenotype can also affect other individuals, such as reducing fitness of breeding partners (Monaghan et al. 2012).

1.2.2 Developmental stress: adverse or adaptive outcomes?

A trait that is adaptive in one context may be maladaptive in a different context. Context is especially important for traits that are irreversibly plastic (i.e., traits that once determined, remain fixed; Stearns 1989) because there could be a mismatch between the future environment and the altered phenotype. The use of environmental cues available to "program" development of a phenotype to match the environmental conditions predicted by those cues underlies many theories addressing the consequences of developmental stress, such as the thrifty phenotype hypothesis and predictive adaptive responses (PARs; Monaghan 2008; Gluckman & Hanson 2004; Hales & Barker 2001).

From the perspectives of the thrifty phenotype and PARs, phenotypic programming by developmental stress is adaptive because these changes serve to help the organism to overcome the immediate threat or to prepare the organism with strategies to deal with future stressful events (Schoech et al. 2011; Love & Williams 2008). Maladaptations resulting from

stress-induced phenotypic changes only occur when the later, adult environment differs from the developmental environment. The maternal programming of stress reactivity in snowshoe hares demonstrates that phenotypic changes in response to developmental stress are not necessarily disadvantageous. High levels of maternal stress hormones are correlated with high predation risk, and offspring of mothers with high stress hormone concentrations exhibited exaggerated endocrine responses to stress (Boonstra et al. 1998; Sheriff et al. 2010). Increased endocrine responsivity to stress is associated with more anxiety and fearfulness (e.g. Kalinichev et al. 2002; Caldji et al. 1998), and factors likely contribute to greater sensory information processing and long-term memory of the event (Badyaev 2005), which in turn potentially improve predator avoidance behaviors. Consequently, hares exhibiting this behavioral and physiological profile may have an advantage if predation risk is high, but may be at a disadvantage if predation risk is low. The main point of this example is that greater responsivity to stress is not necessarily harmful (in that it increases risk for future physical and cognitive problems), but is the price paid by individuals to have greater biological sensitivity to context (Boyce & Ellis 2005).

The impact of maternal environment on stress responsivity and anxiety and fearfulness in snowshoe hares suggests that developmental environments may have programming effects on multiple physiological and behavioral tendencies. Behavioral syndromes (also termed personality, temperament, and coping styles) describe consistent individual differences in various behavioral and/or physiological traits that are stable over time and across contexts (Groothuis & Carere 2005; Stamps & Groothuis 2010). Great tits selected for divergent personalities also demonstrate differences in exploratory behavior, immune function, aggression, testosterone, and HPA axis responsiveness to stress (Stamps & Groothuis 2010; van Oers et al. 2012; Baugh et al. 2012). Reminiscent of the snowshoe hares example, some of these traits appear to be sensitive to programming effects of developmental experience (e.g. Carere et al. 2005; Naguib et al. 2011; Schoech et al. 2011). Thus, developmental experiences can affect the way an individual deals with environmental challenges, potentially by programming the functional interdependence of a set of behavioral and physiological traits.

1.3 DEVELOPMENTALLY CORRELATED TRAITS

Behavioral syndromes are an example of correlations of suites of traits that are influenced by both genetic makeup and developmental experiences. A single environmental factor may affect multiple aspects of a phenotype: for example, nutritional availability may simultaneously affect growth rate and development of morphological structures (Whitman & Agrawal 2009). Correlations between functionally independent traits (such as morphology and cognition; Boogert et al. 2011) may occur if they share developmental schedules and resource requirements (Spencer & MacDougall-Shackleton 2011). Resources available for development may be diverted to overcoming developmental stress and subsequently hinder the development of other traits, resulting in a negative correlation between the traits. Conversely, processes that are prioritized in the face of stress may be positively correlated with each other.

1.3.1 Condition-dependent signals and mate choice

Correlations among traits due to developmental constraints may have influenced the process of sexual selection. In mating contexts, female preference for several and varied aspects of male appearance and courtship displays may have evolved because these different traits signal other aspects of male quality, including current condition, developmental history, and cognitive abilities (Møller & Pomiankowski 1993; Spencer & MacDougall-Shackleton 2011; Boogert et al. 2011; Keagy et al. 2012). Physical traits such as size, coloration, and activity levels may signal current body condition, whereas behavioral traits that have a learned component, such as elaborate songs of songbirds, nest-building skills of weaverbirds, and tool manufacturing skills of crows may signal cognitive capacity (Collias & Collias 1964; Hunt & Gray 2003; Jarvis 2004; Kenward et al. 2006; Walsh et al. 2010, 2011). Larger males with more elaborate display traits may have greater access to food or better foraging abilities, allowing them to invest time and energy into expression of superfluous traits. Thus assessment of these traits may provide females with a way to assess the direct and indirect benefits she may receive from a male, such as greater resources and good genes for her offspring, respectively (Cotton et al. 2004).

Female songbirds tend to prefer males that sing more complex songs, and emerging evidence indicates that song may be an honest signal of male quality because it is related to quality of

other traits (Nowicki & Searcy 2004; Spencer & MacDougall-Shackleton 2012). In song sparrows (*Melospiza melodia*), song repertoire size is correlated with fitness, immune function, body condition, HPA axis function, and over winter survival (Pfaff et al. 2007; Schmidt et al. 2012; MacDougall-Shackleton et al. 2009). Song complexity is also related to associative and spatial learning abilities in other songbird species (Boogert et al. 2008; Pravosudov et al. 2005; Farrell et al. 2012). Song is distinct from other avian vocalizations because it has a substantial learned component and is a plastic trait that can be influenced by developmental stress (Nowicki & Searcy 2004). Development of songs is also restricted to sensitive periods, and for many songbirds it starts soon after hatching and ends at sexual maturity (Brainard & Doupe 2002). Thus, preference for good songs may indicate preference for individuals that were able to buffer the effects of developmental stress on development.

1.4 THESIS OBJECTIVES

The overall objective of this dissertation is to address outstanding issues regarding i) the effects of timing of developmental stress on developmental plasticity, and ii) the extent to which phenotypic plasticity is driven by a change in developmental conditions or by consistently poor developmental conditions without change. In my experiments, I cannot completely separate phenotypic responses to a change in developmental conditions from phenotypic responses to unvaryingly poor conditions. This is because a change in developmental conditions involves a change from poor to good conditions or vice versa. However, my research provides a strong examination of whether consistently poor conditions have different effects than change conditions.

I used nutritional stress to induce poor conditions during development. For the purposes of my dissertation, I defined nutritional stress as conditions that make finding and/or obtaining food more difficult (i.e. food accessibility). I use the zebra finch as a model for my research objectives because captive zebra finches breed readily in laboratory settings, which makes it possible to experimentally manipulate offspring at specific developmental periods. Zebra finches are also songbirds of which song learning and development is extremely well documented. As developmental environments can influence song learning and production, and deficits in song learning cannot be compensated for later in life, zebra finch song may clearly demonstrate the effects of stress during specific periods of life on cognitive development.

1.4.1 Importance of timing of stress for adult physiology and cognition

The timing of stress is critically important in determining the direction of correlation between functionally independent traits that have similar windows of sensitivity to environmental influences (Spencer & MacDougall-Shackleton 2011). Despite the potential importance of timing for the effects of developmental stress on developmental plasticity, few studies in birds have taken this factor into consideration, and those that have usually examined the effect on a single or few variables (see Table 1). Therefore, I investigate the effect of nutritional stress at different developmental periods on different measures of physiological and cognitive function in chapters 2 and 3 of my thesis, respectively. I manipulated food accessibility (i.e. the nutritional stress) before and after zebra finch offspring became nutritionally independent from parents and measured physiological (growth rate, body mass, immune function, body fat, and metabolic rate) and cognitive (associative learning, spatial memory, and exploratory behavior) traits throughout development and in adulthood.

1.4.2 Resource allocation or catch-up growth?

In most studies it is unclear whether the developmental effects of early nutritional stress are mediated by poor nutritional conditions *per se*, or by the rapid, accelerated growth that commonly ensues once nutritional stress ceases. During nutritional scarcity, organisms may preferentially allocate resources to preserve key tissues and to processes necessary for short-term survival, but at the expense of other processes such as growth. However, if nutritional conditions subsequently improve, organisms tend to compensate for earlier growth retardation by accelerating growth. The indirect effect of this compensatory, or catch-up growth, as opposed to the direct effects of poor nutritional conditions, has been suggested to increase the risk of developing many of the adult health-related problems in humans, such as obesity, type II diabetes, and coronary heart disease (Metcalfe & Monaghan 2001; Hales & Ozanne 2003). This issue is difficult to address in human studies because catch-up growth is often confounded by low birth weight, and even in studies where birth weight is controlled, the evidence is correlational (e.g. Beyerlin et al. 2010; Eriksson et al. 2001). In birds, the contributions of possible catch-up growth to the effects of developmental stress on physiology and cognition are also not usually considered. Therefore, the aims of chapters 4 and 5 were to determine whether poor early life nutrition followed by catch-up growth had different effects on the phenotype compared to poor early life nutrition without catch-up

growth. It would have been ideal to isolate the effects of catch-up growth from the effects of poor early life nutrition; however, this was impossible because catch-up growth is defined as a period of accelerated growth following an earlier period of poor nutrition. In chapter 4 I investigate the extent to which catch-up growth has unique effects on learning, behavioral flexibility, and body fat. This chapter also provided an opportunity to replicate the learning experiments in chapter 3. In chapter 5 I examine whether catch-up growth has effects on song learning and production and whether these effects are mediated by the glucocorticoid hormone corticosterone.

Table 1.1 List of studies that manipulate timing of stress or examine catch-up growth

Results of studies that have manipulated timing of developmental stress or have explicitly documented effects of catch-up growth on phenotype. The symbols "−" indicate that treatment reduced trait expression,"+" indicate that treatment increased trait expression, and "none" indicate that treatment did not affect trait expression. Unless specified, studies used zebra finches. Treatment groups are indicated in brackets.

Reference	Manipulation	Timing & groups	Measured traits	Direction of effects
Fisher et al. 2006	Diet quality	hatch - PHD20 (H & L)	Growth rates Associative learning	− L (but with catch-up) − L (if show catch-up)
Criscuolo et al. 2008	Dietary protein quantity	hatch - PHD15; PHD15 - PHD30 (HH, HL, LH, LL)	Growth rates[1] Resting metabolic rate	+ LH (catch-up) + LH
Krause et al. 2009	Dietary protein quantity[2]	hatch - PHD17; PHD17 - 35 (HH, HL, LH)	Resting metabolic rate Body mass loss[3] Exploration & foraging	none + LH − LH
Honarmand et al. 2010	Dietary protein quantity	hatch – PHD17; PHD 17 – 35 (HH, HL, LH)	Biometry[4] Baseline CORT[5] Male cheek patch Survival	+ LH (catch-up) + L conditions none none
Krause & Naguib 2011	Diet quality	PHD3-35 (H & L)	Growth rates[5] Exploratory behavior	− L (but with catch-up) − L (if shows catch-up)
Boogert et al. 2013	Prenatal CORT injections & postnatal food unpredictability[6]	Incubation day 5; PHD4 – 19	Social information use	+ prenatal − postnatal

[1] Calculated using tarsus and wing length with body mass as covariate. [2] Studied in females only. [3] Reduction of body mass after 3 h food deprivation. [4] Biometric traits included tarsus length, wing length, body mass. [5] Calculated as daily body mass gain (g/day) for PHD3-35 and PHD36-168. [6] Studied in Japanese quail (*Coturnix japonica*).

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CHAPTER 2

NUTRITIONAL STRESS AT DIFFERENT DEVELOPMENTAL PERIODS:
EFFECTS ON GROWTH, BODY FAT, BODY MASS, AND IMMUNE FUNCTION
OF ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

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2.1 ABSTRACT

Developmental conditions may influence many aspects of adult phenotype including growth and immune function. Whether poor developmental environments impair both growth and immune function or induce a trade-off between the two processes is inconclusive, and the impact of the timing of stress in determining this relationship has so far been overlooked. We tested the hypothesis that the long-term effects of nutritional stress on growth, body composition, and immune function in zebra finches (*Taeniopygia guttata*) are different dependent whether stress is experienced during an early or juvenile phase (i.e. before or after nutritional independence, respectively). We raised birds on high (H) or low (L) food conditions until post-hatch day (PHD) 35 and switched treatments for half of the birds in each of the H and L groups from PHD 36 – 61. We found that unfavorable juvenile conditions (PHD 36 – 61) increased somatic growth rates, body fat, and some aspects of immune function. We also observed a positive relationship between growth and immune function, as individuals that grew faster as juveniles also had better innate immune responses as adults. There was no effect of treatment on basal metabolic rate. These findings demonstrate the importance of later developmental conditions in shaping multiple aspects of the adult phenotype.

Keywords: developmental stress, adiposity, quantitative magnetic resonance (QMR), bird, phenotypic plasticity

2.2 INTRODUCTION

Poor developmental environments can have short- and long-term consequences on adult phenotype (Birkhead et al. 1999; Lindström 1999; Tschirren et al. 2009). When faced with limited and insufficient resources organisms may prioritize the development and maintenance of particular processes over others, thus inducing a trade-off between these processes. In the short-term, stressful developmental conditions may induce a trade-off between growth and immune function (Fair 1999; Hoi-Leitner et al. 2001; Soler et al. 2003; Brommer 2004; Chin et al. 2005; Brzęk and Konarzewski 2007). In the long-term, there is some evidence that favorable early environments enhance, while unfavorable early environments impair, growth and adult immune function (Birkhead et al. 1999; Tella et al. 2001; Naguib et al. 2004; Stjernman et al. 2008; Butler and McGraw, 2011; De Coster et al. 2011). However, Tschirren et al. (2009) found the opposite pattern, with adult birds raised in larger broods mounting a stronger T-cell-mediated immune response. Råberg et al. (2003) also reported no correlation between nutritional status of nestling blue tits and adult antibody responsiveness. Thus, further investigation is required to resolve the contradiction regarding the persistent effects of developmental conditions on growth and immune function in birds.

One factor that may affect this relationship is the developmental period at which poor environments are encountered. This factor has so far been understudied, even though it may play a critical role in determining the direction of the relationship. Both growth and immune function have prolonged developmental schedules: in more than half the bird species examined by Ricklefs (1968) somatic growth continued through the juvenile period after young fledged the nest. Development of the innate and adaptive components of the immune system also continues well past the nestling stage (Mauck et al. 2005; Strambaugh et al. 2011; Palacios et al. 2009). Conceivably, development and function of physiological processes that are likely to be most affected by early environmental conditions are those that are maturing at that time when substandard conditions are experienced. Thus, growth and development of the immune system may be differentially affected depending on whether poor environmental conditions are experienced early (e.g. nestling phase) or later in development (e.g. juvenile phase).

The developmental period at which stress is experienced is also important because organisms may be able to compensate for detriments incurred during periods of poor conditions if

conditions subsequently improve. Developmentally disadvantaged organisms may accelerate growth to match the size of conspecifics in adulthood, although this "catch-up" growth may have long-term costs (reviewed in Metcalfe and Monaghan, 2001). Additionally, compensation may not occur for all detriments, nor be fully complete, as zebra finches in poor developmental conditions were able to match control subjects on wing length (de Kogel, 1997), but not mass (de Kogel 1997; Tschirren et al. 2009) in adulthood. Thus, further research is needed to understand how the timing of developmental stressors affects growth and immune function, and how this timing influences potential compensation or catch-up growth.

In the current study, we tested the hypothesis that the long-term effects of developmental stress on growth, body composition, and adult immune function in zebra finches are dependent on the developmental period at which stress is experienced. Captive zebra finches were subjected to high (H) or low (L) food conditions, before or after nutritional independence. We then measured basal metabolic rate, body fat percentage, immune function, and organ mass of the same birds as adults. Our results demonstrate that poor developmental conditions differentially affect adult phenotypes depending on the stage of development they occur, but that unfavorable conditions experienced after nutritional independence have the strongest effect on growth, adult body condition, and immune function.

2.3 METHODS

2.3.1   Animals and Manipulation

We randomly paired adult male and female zebra finches from our breeding colony in June 2011. Each pair was housed in a 36 x 43 x 42 cm cage with access to an external nest box (20 x 13.5 x 13.5 cm) and kept on 14L:10D light: dark cycle at 22˚C. Pairs received grit, cuttlefish bone, seed (Living World premium finch seed; 11.0% protein, 5.9% lipid), and water ad libitum, and were supplemented with daily portions of egg-food (hardboiled chicken eggs, cornmeal, bread). All animal care and husbandry protocols were approved by the Animal Use Subcommittee at Western (#2007-089), and followed guidelines of the Canadian Council on Animal Care. We used a total of 9 pairs that produced 33 experimental offspring in this study. Non-independence of nest-mates was controlled statistically (see below).

Nests were monitored daily for nesting activity and randomly assigned to treatment conditions after the first egg hatched.

Experimental treatment began when the oldest nestling was 6 days post-hatch (PHD6) and lasted until approximately PHD 61. Only broods with 4 or 5 nestlings at the start of treatment were included in our experiment. We manipulated food accessibility (Spencer et al. 2003), where broods in the high feeding treatment (H) were given access to 65g seed and 13.5g egg-food daily, while broods in the low feeding treatment (L) were given access to 50g total of seed in a mixture containing a 1:3 ratio of seeds and woodchips (by volume), and 6.5g egg-food daily. This treatment forces parents in the L treatment to search longer for seeds from the time treatment starts until offspring reach PHD 35 and become nutritionally independent. Similar feeding treatments have been shown by Lemon (1993), Spencer et al. (2003), Buchanan et al (2004), and Zann and Cash (2008) to negatively affect body mass, adult song control brain regions, and song characteristics of zebra finches raised in these conditions.

Our experiment was designed to test effects of feeding treatment during two phases: an early (PHD 6 – 35, before offspring were nutritionally independent) and juvenile phase (from PHD 36 – 61, after offspring were nutritionally independent). We raised zebra finches on high (H) or low (L) food conditions during the early phase and then switched feeding treatments for half of the birds in each of the H and L groups during the juvenile phase. This resulted in four feeding treatments: HH, HL, LH and LL. After the juvenile phase, all birds were given *ad libitum* seed. Offspring were kept with their parents until PHD 90 to ensure that young males learned song from their fathers exclusively (Adret 1993; song data was collected for chapter 5) and then housed in same-sex groups of four to five birds.

2.3.2 *Growth and Body Fat*

To calculate growth, we weighed subjects daily using an electronic scale accurate to 0.1g from hatch (PHD 1) to approximately PHD 65. Growth rates (k) were defined as [log mass$_2$ – log mass$_1$]/[t$_2$-t$_1$] (Fisher et al. 2006; Morton et al. 1985). We calculated growth rate constants (k) for the each of the treatment phases (i.e. early and juvenile phase).

Body fat was quantified on approximately PHD 25, 50, and 59, 180 and 600 using quantitative magnetic resonance (QMR) body composition analysis, using an instrument

designed for small birds (model MRI-B; Echo Medical Systems, Houston, TX). With QMR, we are able to obtain a more comprehensive measure of body condition, as we can examine both total mass, and percentage of body fat in proportion to total body mass. This QMR analysis has been shown to accurately and precisely detect lean and fat mass in various bird species including zebra finches (Gerson and Gugliemlo 2011; Guglielmo et al. 2011), and here we report the values accurate to 0.01g. The QMR analyzer was calibrated with 5 or 2.1g of canola oil before each measurement. Coefficients of variation for fat and lean mass are 3% and 0.5%, respectively, and relative accuracies are ±11% and ±1%, respectively (Guglielmo et al. 2011). Lean and fat content were adjusted (raw value x 0.94 for fat mass, raw value x 1.021 for lean mass) according to calibration equations for zebra finches (Gerson and Guglielmo 2011; Guglielmo et al. 2011).

2.3.3   Basal Metabolic Rates

We measured basal metabolic rates (BMR) when birds were approximately PHD 180, using a flow through respirometry system similar to Gerson & Guglielmo (2011). Birds were weighed and placed into individual well-sealed respirometry chambers overnight and maintained at a constant temperature of 35˚C, which is within the thermoneutral zone for zebra finches (Calder 1964). We recorded five birds per night, and the baseline was taken from a channel sampling air within the room. Incurrent air was scrubbed of CO_2 and water vapor using soda lime and Drierite, respectively, and the chambers received a constant flow of 350 mL/min. Birds were fasted for 3 h in the chambers, and only data from the remaining 7 h of the night were used to ensure that measurements would reflect metabolic rates of birds in post-absorptive state. Excurrent air was subsampled at a rate of 150 mL/min, and was passed through a Drierite column to the CO_2 (CA-2A; Sable Systems, Las Vegas, NV) and O_2 analyzers (Sable Systems FC-1B), with CO_2 and H_2O scrubbing between the analyzers. Gas analyzers were calibrated with a certified standard containing 20.9% O_2-1.0% CO_2 balanced with N_2 (Praxair, London, Canada). Multiplexing enabled measurements of a 10 min baseline and 10 min samples from each chamber every hour. All instruments were connected to an analog-to-digital converter (UI-2 model, Sable Systems), which was connected to a laptop computer. Expedata software (Sable Systems) was used for both data collection and analysis. We corrected for lag time between O_2 and CO_2 measurements, and used equations 10. 6 and 10.7 in Lighton (2008) to calculate V_{O_2} (mL/min) and V_{CO_2}

(mL/min) based on the mean of the final 5 min of each 10 min sampling interval (Gerson and Guglielmo 2011). We also calculated the respiratory quotient (RQ) as V_{CO_2}/V_{O_2} (Lighton 2008). The RQ value indicates energy production resulting from pure lipid catabolism to pure carbohydrate catabolism, with values ranging from 0.7 to 1.0, respectively.

2.3.4   Immune Function

Innate constituent immunity: The anti-microbial capability of blood from each subject was assessed with microbe killing assays against a strain of *Escherichia coli* (ATTC #8739; Epower Microorganisms, catalog #0483E7, MicroBiologics) and *Candida albicans* (ATTC #10231; Epower Microorganisms, catalog #0443E7, MicroBiologics). Killing of *E. coli* is dependent on complement proteins while killing of *C. albicans* is dependent on interactions between plasma factors and phagocytosis (Millet et al. 2007). We first conducted optimization assays to determine optimal microbe concentration, blood to cell media dilution, and incubation times following procedures outlined by Liebl and Martin (2009). For optimization of the *C.albicans* assay, we reconstituted the lyophilized pellet of *C.alibcans* and diluted the stock with sterile PBS to obtain a working solution of 1 x 10^4 CFU/mL. We then incubated the *C.albican* working solution in a 1:24 dilution of fresh blood to cell media for 15 min. For optimization of the *E.coli* assay, we reconstituted the lyophilized pellets of microorganisms according to the manufacturer's instructions and diluted the stock concentration with sterile phosphate buffered saline (PBS) to obtain a working solution of 1 x 10^5 CFU/mL. We then incubated the *E. coli* working solution in a 1:6 dilution of previously-frozen whole blood to cell media for 30 min (see below for details).

When birds were approximately PHD 150, sterile blood samples were collected in less than 5 min from the brachial vein. We first cleaned the skin with cotton balls soaked with 70% ethanol, waited for the ethanol to dry, collected the blood with sterile heparinized capillary tubes, and then transferred the blood into sterile microcentrifuge tubes. A portion of the fresh blood was immediately aliquoted out into separate sterile microcentrifuge tubes containing cell media (CO2-Indepenedent media, Invitrogen #18045-088; L-glutamine, Sigma-Aldrich; fetal bovine serum, Invitrogen) for the *C. albicans* assay. The remaining whole blood was stored at -80˚C for the *E. coli* assay and assayed within 10 days of sample collection.

For each assay, we made three replicates of an assay positive control (APC) and two replicates of an assay blank control (ABC). APCs contained microbes but no blood (36uL sterile PBS, 12.5uL working solution, 250uL tryptic soy broth). Consequently, APC values reflect unhindered bacterial growth, and were used to ensure that microbe concentrations reached a particular absorbance range for spectrophotometry. Too low APC absorbance values would have made it difficult to detect small absorbance difference between samples. On the other hand, ABCs contained no blood or microbes (48.5uL sterile PBS and 250uL tryptic soy broth). ABCs were used as basal values for APCs to account for any effects on absorbance that were not due to bacterial growth. Furthermore, each subject had an individual blank control (IBC) to account for individual variation in blood coloration. IBCs contained blood, but no microbes (36uL blood, 12.5uL sterile PBS, 250uL tryptic soy broth). Antimicrobial activity was assessed by subtracting the absorbance of the same subjects' IBC from the absorbance of a subject's sample (i.e. absorbance of each sample was adjusted according to the corresponding IBC). All controls (APC, ABC, and IBC) were made alongside samples and incubated with samples to ensure that all tubes were exposed to the same conditions.

Microbe killing assays for *C. albicans* commenced immediately after sample collection. For each individual bird, we had three sterile microcentrifuge tubes and each tube contained 36 µL of a 1:24 dilution of blood to cell media. Two of the tubes were sample replicates and the other served as the individual blank control. We made a working solution of 1×10^5 CFU/mL, added 12.5 µL of the working solution to the samples, vortexed all tubes thoroughly, and incubated them at 30˚C for 15 min. We then vortexed and added 250 µL tryptic soy broth (product #1.05459; EMD Chemicals) to all tubes. Samples were then incubated at 30˚C for 24 – 48 h, after which the absorbance (abs) of each sample was measured using a Nanodrop spectrophotometer (Nanodrop 2000c; Thermoscientific). We used the average value of sample replicates in our calculations. Percentage of microbes killed in each sample was quantified as *(abs_{sample}-absIBC/absAPC-abs_{ABC})* x 100. Quantifying antimicrobial activity as the proportion of microbes killed in samples relative to APCs accounted for any differences in secondary incubation times between assays.

Similar procedures were used in the microbe killing assays for *E. coli*. For each individual, we had three sterile microcentrifuge tubes, each containing 36uL of a 1:6 dilution of previously-frozen blood to cell media. We then made a working solution of 1×10^4 CFU/mL, added 12.5uL of the working solution to all samples, vortexed and incubated them at 37˚C for 30 min. We then vortexed samples, added 250 uL tryptic soy broth, and incubated samples at 37˚C for 12 h. Absorbance readings of samples were then taken using the Nanodrop spectrophotometer and percentage of microbes killed in each sample was quantified as *(abs$_{sample}$-absIBC/absAPC-abs$_{ABC}$)* x 100

Adaptive induced immunity: Our aim was to assess effects of treatment on subjects' ability to mount an immune response to a foreign novel antigen. We used a haemagglutination assay to assess levels of antibody-mediated agglutination after exposure to sheep red blood cells (SRBC; Deerenberg et al. 1997; McGraw & Ardia 2005). We did not distinguish between SRBC-specific and non-specific natural antibodies in because we were not interested in differences in production of specific antibodies.

We first diluted a stock of 10% suspension of SRBC (catalog #55876; MP Biomedicals) to 2% with sterile PBS. When birds were approximately PHD 360, they were injected intraperitoneally with 100 µL of 2% SRBC using an insulin syringe with a 29 gauge needle. Blood samples were again taken 6 days later to assess primary antibody response (Birkhead et al. 1998). Plasma was extracted after centrifugation at 13000 g for 10 min, stored at -80˚C, and assayed within 10 days of collection.

For the assay we followed procedures described by Matson et al. (2005). In 96-well round-bottom microtitre plates, we serially diluted 20 µL of plasma in 20 µL of PBS (1:1 dilution). Each plate also had a negative control (PBS only) and positive control (chicken plasma). We then added 20 µL of 1% SRBC to all wells and incubated the plates at 37˚C for 90 min. After incubation the plates were rested on a tilted stand for 20 min and then scanned with a flatbed scanner (Epson Perfection 4990 Photo) at 300dpi with the "positive transparency" setting. An experimenter blind to treatment conditions scored a plate by comparing haemagglutination of the samples to that of the positive and negative controls on the plate. Haemagglutination scores were expressed as log$_2$ of the highest dilution showing agglutination.

2.3.5   Statistics

Statistical analyses were conducted using SPSS 19.0. We used linear mixed models with restricted maximum likelihood (REML) to analyze the effect of treatment on all our dependent measures (i.e. growth, body fat, adult body mass, metabolic rates, immune function). This analysis is appropriate for our data because we can control for the potential nonindependence of our samples (i.e. relatedness of siblings in each nest) to avoid pseudoreplication. In all of the analyses described below we first tested the significance of the random effects of broods and individuals nested in broods by using maximum likelihood theory to compare fitting of data into a similar model without the random effect. As these random effects did not contribute significantly to all models, some results are reported with these random effects excluded from the final model. All two-way and higher-order interactions were included in the full model and stepwise deletion of non-significant terms was applied to obtain the most parsimonious model of the data. Pairwise comparisons between treatment groups were adjusted using Sidak corrections.

Growth Rates: We calculated growth rate constants (k; following Morton et al. 1985) for the early and juvenile phase. We analyzed each phase using separate linear mixed models, with growth rate k as the dependent variable, feeding treatment (HH, HL, LH, LL), sex as fixed effects. No random effects were retained in the final model. We attempted to control for parental effects by including parent mass (average of mother and father mass) as a fixed covariate, but it did not significantly contribute to the model and was removed.

Body fat: To examine effects of feeding treatment on body fat, grams of body fat was entered as the dependent variable, with feeding treatment (HH, HL, LH, LL), age (PHD 25, 50, 59, 180, and 600), sex as fixed effects, and with total mass minus fat mass as a fixed covariate (Christians, 1999). Individuals nested in broods were retained as a random effect in the final model.

Adult body mass: To examine effects of feeding treatment on body mass, we entered body mass (g) of birds at PHD 600 as the dependent variable, with feeding treatment (HH, HL, LH, LL) and sex as fixed effects. No random effects were retained in the final model.

Metabolic Rates: To analyze effects of feeding treatment on metabolic rates, BMR (Watts) was entered as the dependent variable, with feeding treatment (HH, HL, LH, LL) and sex as

fixed effects, and with body mass as a fixed covariate. For RQ, the RQ value was used as the dependent variable and all fixed effects and covariates were identical to BMR. For both BMR and RQ, no random effects were retained in the final model.

Immune Function: To analyze effects of feeding treatment on innate constitutive immune function, we ran separate linear mixed models for microbicidal capacity against *C.albicans* and *E.coli.* For both analyses % killing was the dependent variable, with feeding treatment (HH, HL, LH, LL) and sex as fixed effects. No random effects were retained in the final model. For adaptive induced immunity, we entered agglutination scores as the dependent variable, with feeding treatment (HH, HL, LH, LL) and sex as fixed effects. Random effects were excluded from the final model.

Relationship between growth and immune function: To analyze whether growth rates during development influenced adult immune function, separate linear mixed models were used for each of the dependent variables (% killing *E.coli*, % killing *C.albicans*, and haemagglutination score), with feeding treatment (HH, HL, LH, LL) as fixed effects, and with early and juvenile growth rates (k) as covariates. Brood ID and individual ID were included in the final model. Only interactions between main-effects were considered in these models.

Table 2.1 summarizes the dependent variables, fixed and random effects entered into the linear mixed models for measures of growth, body fat, immune function, body mass, and metabolic rates.

2.4 RESULTS

A total of 33 offspring zebra finches from 9 different broods were successfully reared according to our feeding treatments (HH = 6, HL = 10, LH = 9, LL = 8). We had relatively equal numbers of females and males for each feeding treatment, except for HH that only had one male (HH females = 5, males = 1; HL females = 5, males = 5; LH females = 4, males = 5; LL females = 4, males = 4). Therefore, any significant feeding treatment × sex results that involves the HH group should be interpreted with caution.

Table 2.1. Summary of fixed and random effects of the linear mixed models for analysis of growth rates, body fat, adult body mass, immune function, and metabolic rate

Significant random effects that were included in the final model are marked with an asterisk (*). The term individuals nested in broods is abbreviated as indiv(brood). Fixed covariates are italicized.

	Dependent variable	**Fixed effects**	**Random effects**
Growth rate (early and juvenile phase)	growth constant k^1	feeding treatment sex *parent mass²*	brood indiv(brood)
Body fat	body fat (g)	feeding treatment sex *age³* *total mass – fat mass*	brood indiv(brood)*
Adult body mass	PHD 600 mass (g)	feeding treatment sex	brood indiv(brood)
Innate immunity (*C.albicans* and *E.coli*)	% killing	feeding treatment sex	brood indiv(brood)
Humoral immunity	haemagglutination score	feeding treatment sex	brood indiv(brood)
Metabolic rate	BMR (Watts)	feeding treatment sex *body mass*	brood indiv(brood)
	RQ	feeding treatment sex *body mass*	brood indiv(brood)

[1] Growth rates calculated following Morton et al. (1985). [2]Parent mass (average of mother and father mass) was non-significant and removed. [3]Values for age were PHD 25, 50, 59, 180, 600.

2.4.1 Growth rates

Our results indicate that feeding treatment significantly affected growth rates only during the juvenile phase. Growth during the early phase was not significantly affected by feeding treatment or sex (feeding treatment $F(1, 25) = 1.57$, $p = 0.222$; sex $F(1, 25) = 0.28$, $p = 0.599$; figure 2.1a). On the other hand, growth during the juvenile phase was significantly affected by feeding treatment ($F(3, 28) = 12.73$, $p < 0.001$), but did not differ between sexes ($F(1, 28) = 0.010$, $p = 0.922$). Pairwise comparisons of the main effect of feeding treatment indicated that HL conditions increased growth rates compared to HH conditions and LL conditions ($p < 0.001$ and $p = 0.002$, respectively). Similarly, LH conditions also elevated growth rates compared to HH conditions ($p = 0.003$; figure 2.1b). In short, birds that experienced a diet switch in juvenile phase grew significantly faster than birds that were maintained on the same diet as the early phase. Therefore it appears that a change (either an improvement or deterioration) in nutritional conditions stimulates faster growth in developing offspring zebra finches.

2.4.2 Body fat

Feeding treatment during development had significant effects on body composition. Body fat was significantly affected by feeding treatment and age (feeding treatment $F(3, 23.01) = 4.55$, $p = 0.012$; age $F(4, 102.72) = 10.88$, $p < 0.001$), but not sex ($F(1, 22.70) = 0.00025$, $p = 0.99$). Fat mass also covaried significantly with total mass minus fat mass ($F(1, 35.44) = 11.07$, $p = 0.002$), where birds that had more fat mass also had more non-fat mass ($t(35.44) = 3.33$, $p = 0.002$). Pairwise comparisons of the main effect of feeding treatment indicated that HL conditions significantly increased body fat compared to HH and LH conditions ($p = 0.042$ and 0.032, respectively; figure 2.2a). Pairwise comparisons of the main effect of age indicated that birds had more body fat as adults (PHD180 and 600) than during development (PHD 25, 50, and 59; $p < 0.05$ for all; figure 2.2b). These results indicate that nutritional conditions during development can have long-lasting consequences on body fat, with a switch from H to L conditions causing the greatest fat accumulation.
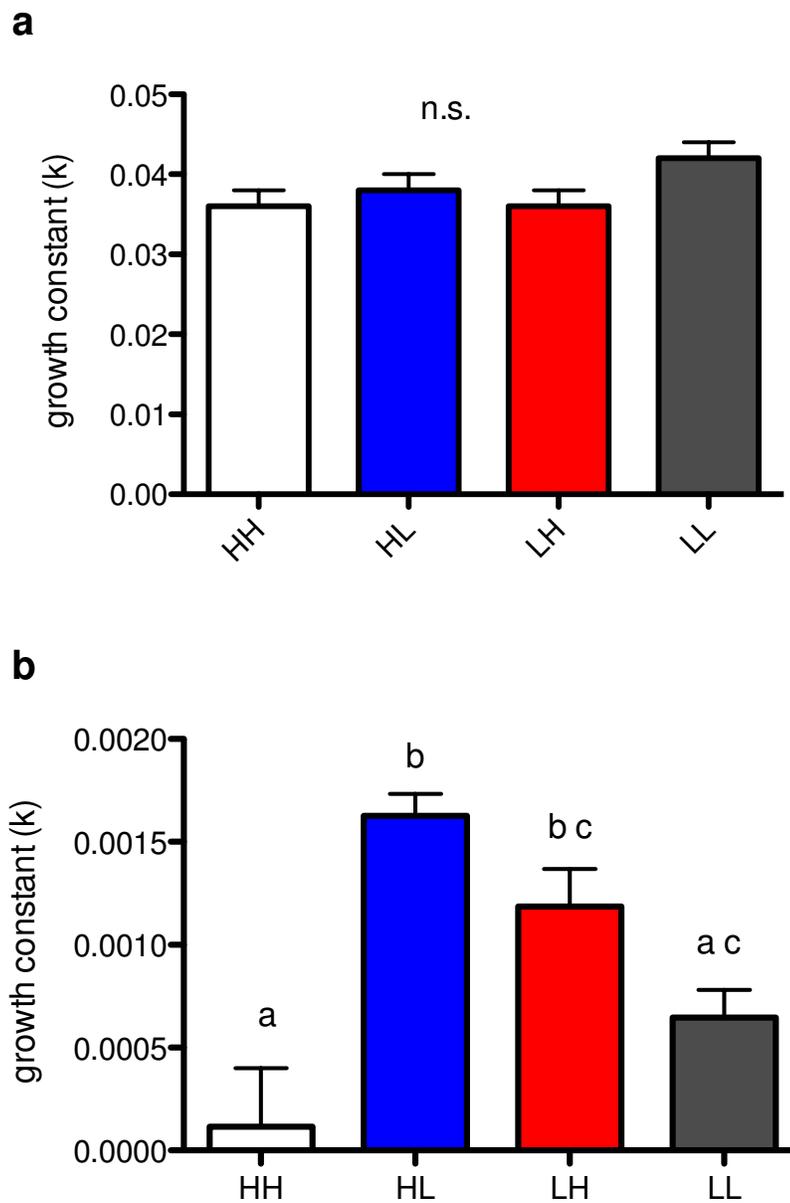
Figure 2.1. Growth rates during the early and juvenile phase

Feeding treatment did not significantly affect growth rates during the early phase (a) but did significantly affect growth rates during the juvenile phase (b). In the juvenile phase, birds that experienced a change in feeding treatments (i.e. HL and LH groups) grew faster than birds that did not experience a change in feeding treatments (i.e. HH and LL). Error bars are ± 1 SEM.
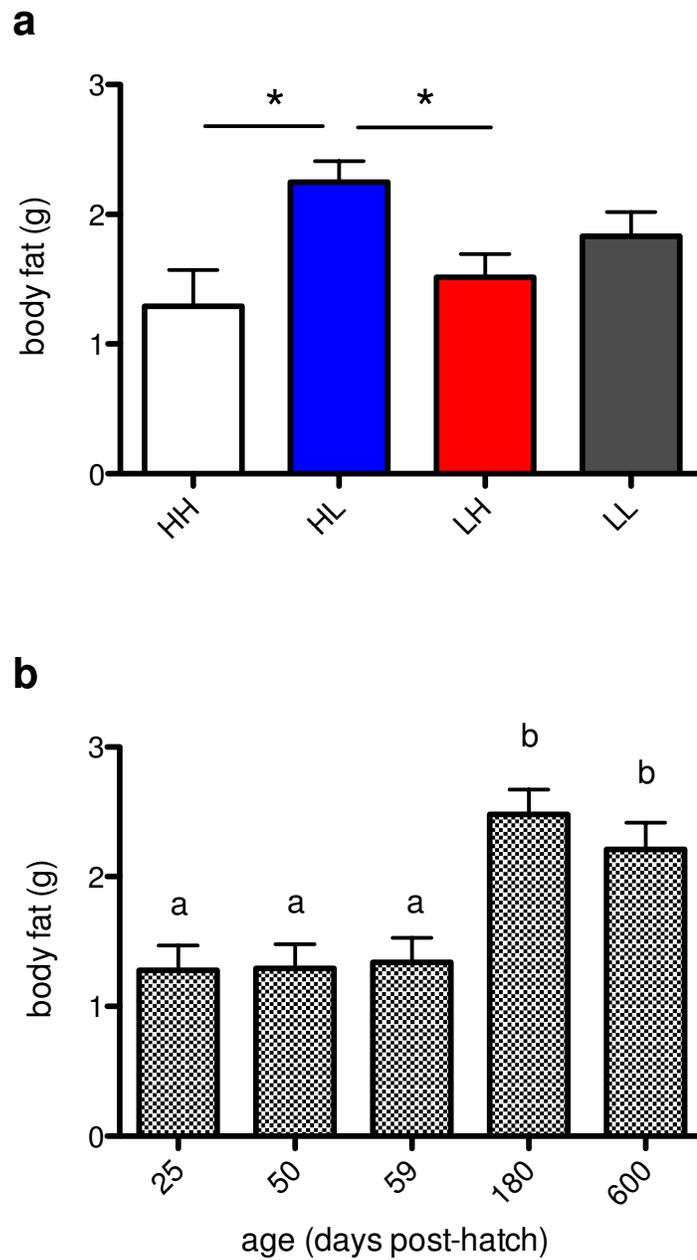
Figure 2.2. Main effects of feeding treatment and age on body fat

Body fat was significantly affected by feeding treatment. The HL group had significantly more body fat than the HH and LH groups (a). Body fat also changed significantly with age. Birds had more body fat as adults than during development (b). Error bars are ± 1 SEM.

2.4.3   Adult body mass

Our results suggest that adult body mass at PHD 600 was affected by treatment conditions during development in a sex-specific manner. Adult body mass was significantly affected by feeding treatment ($F(3, 22) = 3.20$, $p = 0.043$), but not by sex ($F(1, 22) = 0.019$, $p = 0.892$). Adult body mass was also significantly affected by the interaction of feeding treatment × sex ($F(3, 22) = 6.32$, $p = 0.003$). No pairwise comparisons of the main effect of feeding treatment were significant with Sidak corrections (figure 2.3a), however, analysis without correction for multiple comparisons indicated that LH conditions reduced body mass compared to HL and LL conditions ($p = 0.014$ and 0.020, respectively). Pairwise comparisons of the interaction indicated that HL males were significantly heavier than HH males and LH males ($p = 0.042$ and 0.002, respectively; figure 2.3b). Feeding treatment did not significantly affect female adult body mass. These findings indicate that nutritional stress during development can have long lasting and sex-specific consequences for body mass.

2.4.4   Basal metabolic rate

BMR and RQ were not significantly affected by feeding treatment (BMR $F(3, 24) = 0.55$, $p = 0.652$; RQ $F(3, 24) = 0.81$, $p = 0.501$; data not shown) or by sex (BMR $F(1, 24) = 0.45$, $p = 0.509$; RQ $F(1,24) = 0.066$, $p = 0.799$; data not shown).

2.4.5   Innate constitutive immunity

Feeding treatment during development significantly affected the ability of components in blood to kill microbes when subjects were adults. Ability of blood to kill *C.albicans* was significantly affected by feeding treatment ($F(3, 25) = 4.76$, $p = 0.009$), but not by sex ($F(1, 25) = 0.55$, $p = 0.465$). Birds that experienced HL conditions killed significantly more *C.albicans* than birds that experienced LH conditions ($p = 0.013$; figure 2.4a). Ability of blood to kill *E.coli* was also significantly affected by feeding treatment ($F(3, 22) = 5.21$, $p = 0.007$) and the interaction of feeding treatment × sex ($F(3, 22) = 3.334$, $p = 0.038$), but not by sex ($F(3, 22) = 3.334$, $p = 0.038$). Birds that experienced HL conditions killed significantly more *E.coli* than birds that experienced LL conditions ($p = 0.012$) and almost significantly more than HH ($p = 0.051$; figure 2.4b). Pairwise comparison of the feeding treatment × sex
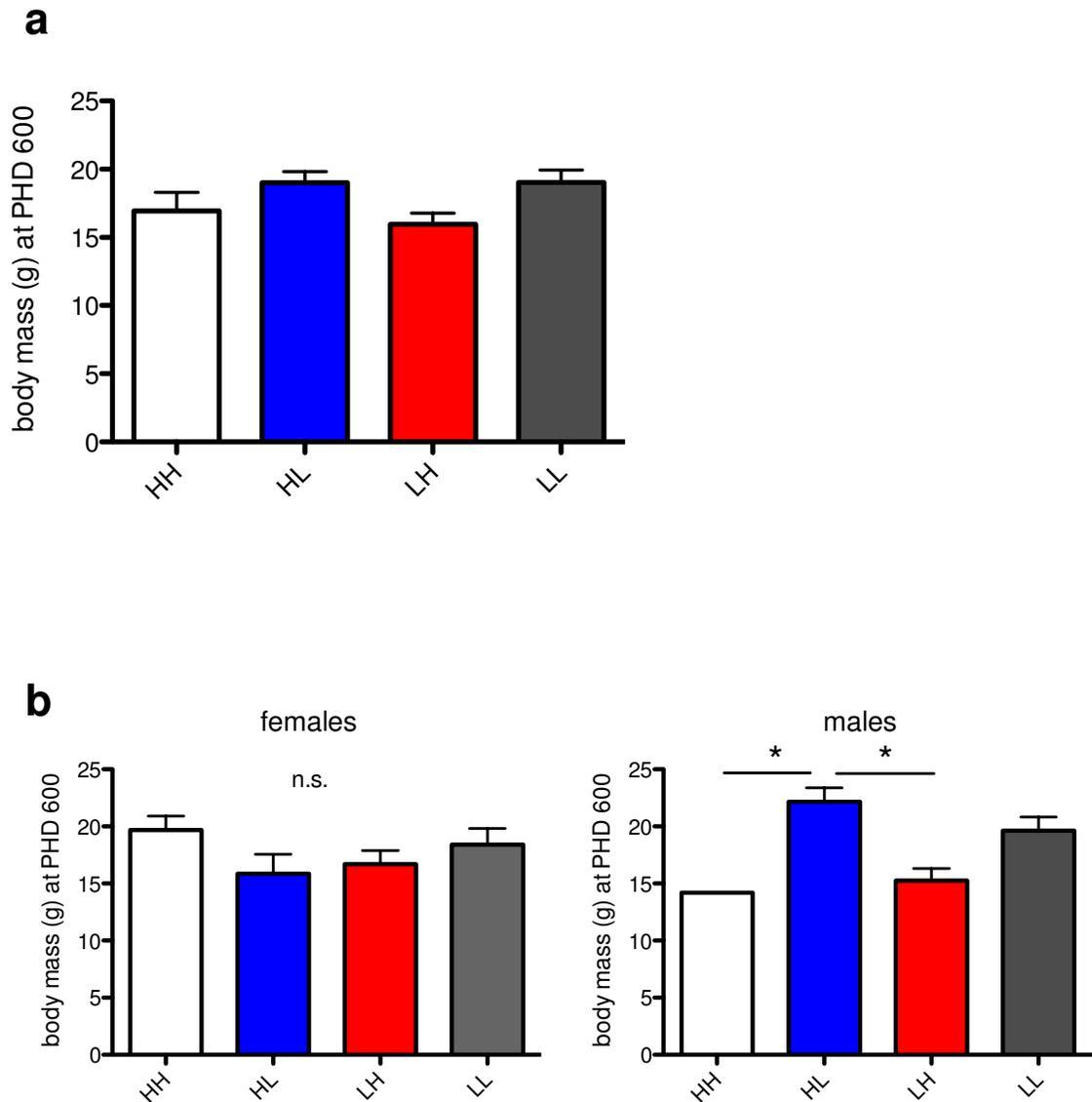
Figure 2.3. Main effect of feeding treatment and feeding treatment × sex interaction on body mass

Body mass was not significantly affected by feeding treatments (a). However, body mass was significantly affected by the interaction of feeding treatment × sex. HL males weighed significantly more than HH and LH males, but females in the different groups did not differ in body mass (b). Error bars are ± 1 SEM.
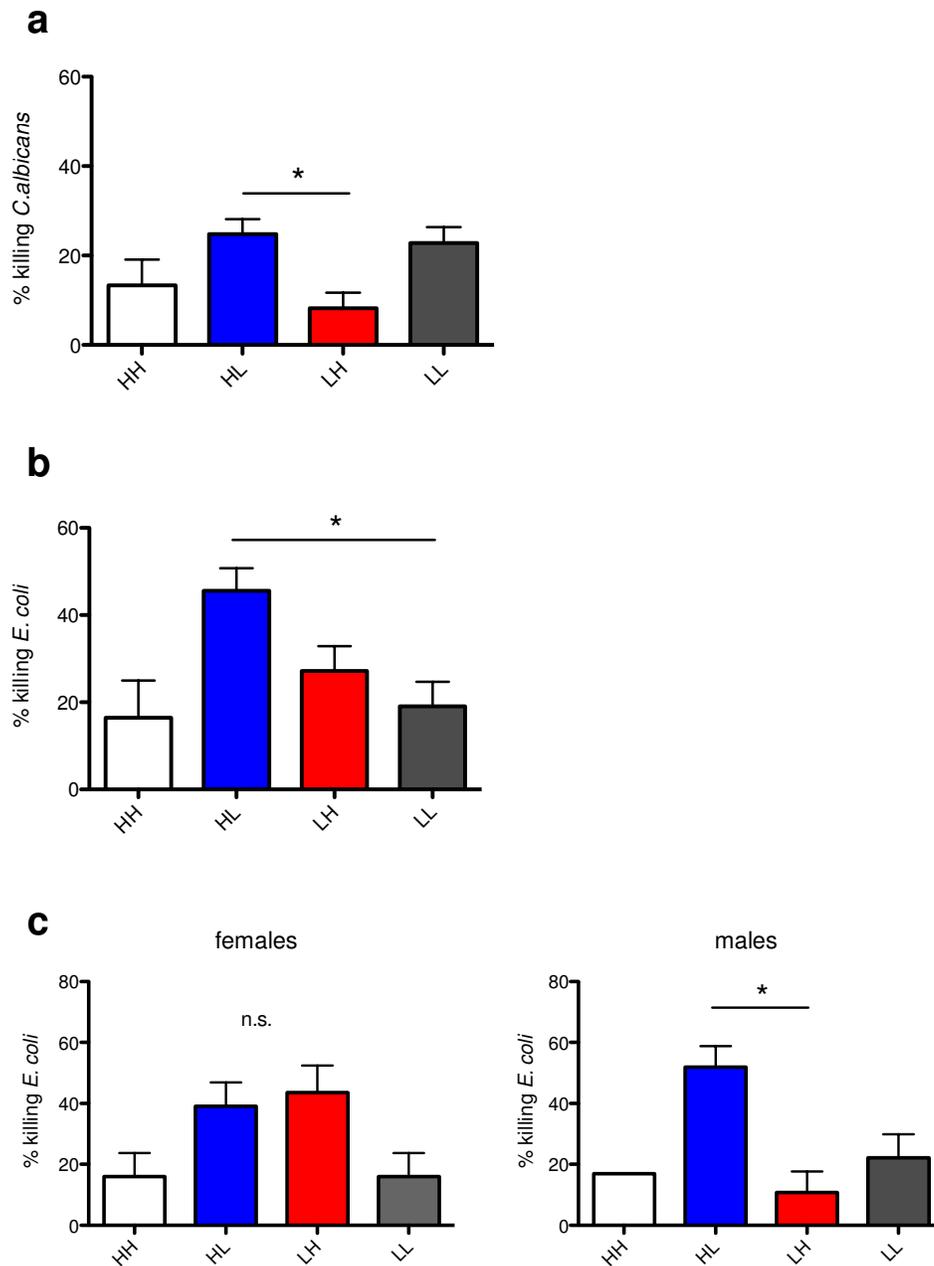
Figure 2.4. Main effect of feeding treatment on *%*killing of *C.albicans* and *E.coli*, and the interaction of feeding treatment × sex on *%*killing *E.coli*

Innate immune function was significantly affected by feeding treatment. Birds in the HL group killed significantly more *C.albicans* and *E.coli* than LH and LL, respectively (a). HL males also killed more *E.coli* than LH males (b). Error bars are ± 1 SEM.

interaction indicated that males that experienced HL conditions killed significantly more *E.coli* than males that experienced LH conditions ($p = 0.002$) and almost significantly more than LL ($p = 0.052$; figure 2.4c). Feeding treatment did not significantly affect females' ability to kill *E.coli* . These results indicate that switching from H to L conditions enhances innate constitutive immune function.

2.4.6 *Adaptive induced immunity*

Feeding treatment did not seem to affect adult humoral immune function. Analysis of haemagglutination after exposure to SRBC revealed no significant main effect feeding treatment or sex ($F(3, 4.64) = 0.37$, $p = 0.779$ and $F(1, 22.40) = 1.29$, $p = 0.269$, respectively; data not shown).

2.4.7 *Relationship between growth and immune function*

Our results suggest that there is a relationship between growth rate during a specific period of time and immune function. We did not find that growth rates (k) in the early or juvenile phase covaried significantly with any of our immune measures. This suggests that effects of feeding treatment on immune function were not mediated by differences in growth rates, so we collapsed data across feeding treatments (i.e. removed feeding treatment as a grouping variable) and ran separate linear multiple regressions, using each of the three immune measures as dependent variables, and using early and juvenile growth rates (k) as the independent variables.

Using multiple regression, we found that growth rates during development significantly predicted ability to kill *E.coli* as adults ($F(2, 27) = 4.12$, $p = 0.028$, $R^2 = 0.234$, $N = 30$). Growth rates during the early phase did not significantly predict ability to kill *E.coli* ($\beta = -0.005$, $t(29) = -0.027$, $p = 0.979$), but growth rates during the juvenile period did ($\beta = 0.482$, $t(29) = 2.67$, $p = 0.013$). This indicates that faster growth during the juvenile phase positively predicted ability to kill *E.coli* as adults (figure 2.5). However, ability to kill *C.albicans* and haemagglutination scores were not predicted by growth rates. Therefore, these results suggest that faster growth during development can be positively correlated with specific aspects of immune function.
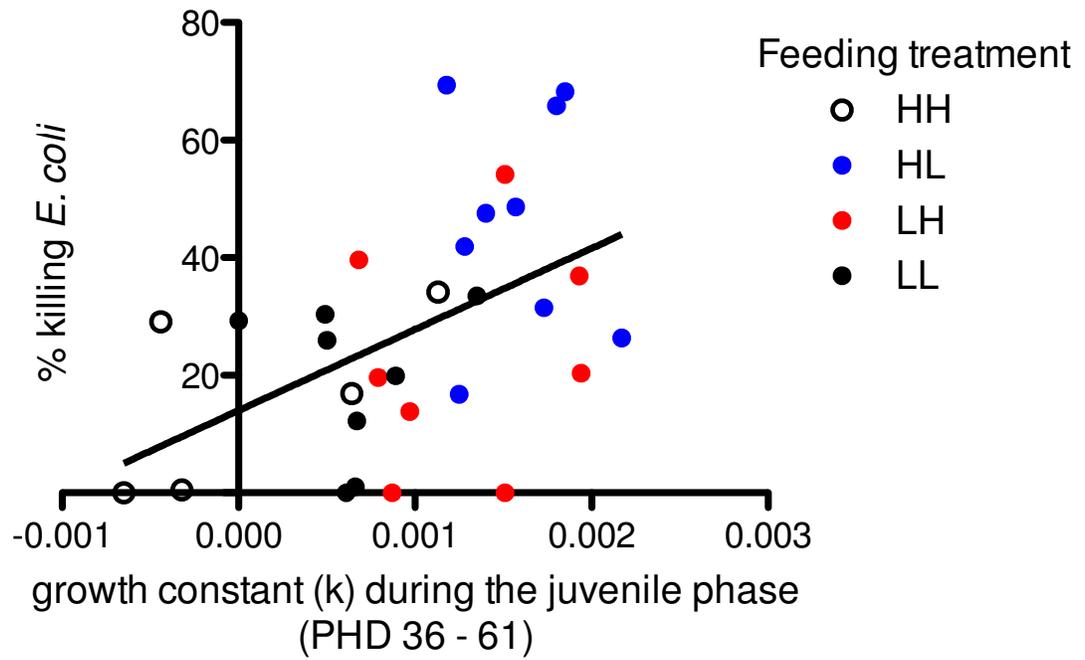
Figure 2.5. Simple regression of juvenile growth rates and *%kiling E.coli*

Growth rates during the juvenile phase positively predicted ability to kill *E.coli* in adulthood.

2.5 DISCUSSION

Our study demonstrates that growth, body fat, and immune function can be differentially affected by the period at which developing organisms face adverse environments. We found that a switch from H to L conditions may cause birds to preferentially invest in traits such as increased somatic growth, fat deposition, and immune function that likely prepare them for suboptimal adult conditions. In the broader perspective, these results provide further evidence of phenotypic programming, where conditions during development may modify or guide development towards a particular phenotype that is better adapted for a particular environment (Monaghan 2008).

Our results also highlight the importance of the developmental period at which stress is experienced in determining the nature of the relationship between growth and immune function. Developmental correlations between such traits will depend on the timing of the stressor and on changes in the susceptibility of developing systems to the stressor (Spencer and MacDougall-Shackleton 2011). Previous studies on the long-term effects of early developmental environments on adult immune function were inconsistent, with some suggesting that poor early environments enhance immune activity, while others suggested the opposite (e.g. Naguib et al. 2004; Stjernman et al. 2008; Tschirren et al. 2009). This inconsistency reveals the complex nature of the relationship between growth and development of immune function: there may be developmental periods when organisms must face a trade-off between growth and immune function, but there may also be other periods when both processes are enhanced or impaired by a stressor. Furthermore, the trade-off or enhancement may affect the different arms of the immune system to varying degrees.

Although our data do not support the hypothesis that there is a trade-off between early growth and immune function (Lochmiller and Deerenberg 2000), they do suggest that faster growth at particular developmental periods may be associated with enhancement of specific aspects of immune function. Our findings indicate that faster growth during the juvenile period leads to better adult innate immune responses against *E.coli*. Organisms may invest in innate immune defenses (especially when growing up in unfavorable conditions) because these defenses are inexpensive to develop and maintain (Lee 2006) and vital for determining survival and fitness (Lochmiller and Deerenberg 2000). With poor conditions during the juvenile phase (i.e. after nutritional independence) resulting in faster growth, increased

immune defenses, and elevated adult body fat, our results point to a life-history strategy where experiencing impoverished environments later in development primes the organism to favor immune defenses that are crucial for survival, but that are non-specific and inexpensive to maintain in order to invest resources into other physiological processes that increase fitness (perhaps reproduction). As poor maternal environments can result in greater offspring parasite resistance potentially due to increased maternal investment (Boots and Roberts 2012), a future question of interest is whether developmental stress can have a trans-generational effect: birds that invested more in immune function because they experienced developmental stress may also invest more in the immune response of their offspring.

Surprisingly, birds that experienced a switch in feeding treatments grew faster during the juvenile phase than birds that experienced consistent feeding treatments. The faster growth of the LH birds may have been due to catch-up growth, which is a common phenomenon whereby organisms that encounter improved conditions after previously experiencing impoverished conditions will accelerate growth. This rapid catch-up growth allows organisms to compensate for reduced body size incurred during impoverished conditions. Despite these advantages, catch-up growth can also be costly (Metcalfe & Monaghan 2001). Our results suggest that LH birds may have sacrificed adult innate immune function for juvenile growth because HL birds that experienced a switch in the opposite direction actually showed enhanced immune function.

The experimental manipulation did not produce the expected differences in growth rates in the early phase, which contradicts results from other studies that have used the same experimental methods (e.g. Lemon 1993; Spencer et al. 2003; Zann & Cash 2008), although they measured growth as differences in mass at particular ages instead of calculating growth rate constants as we did. Possible explanations for the lack of differences in growth rate in early life are: (1) parents buffered their offspring from poor nutritional conditions at the expense of self-maintenance, (2) the treatment conditions were not stressful enough, or (3) body mass is strongly genetically determined. The first explanation is plausible, as studies have found that parents may buffer the costs of reduced resource availability to the offspring (Mauck and Grubb 1995; Moreno et al. 1999), to themselves (Saino et al. 1999; Takahashi et al. 2003; Ardia 2005), or they may share the costs equally with offspring (Gaston and Hipfner 2006). Future experiments should therefore monitor parental condition in order to

avoid the confounding effects of parental care. The second explanation is improbable as we observed significant effects of feeding treatment on other physiological parameters. More likely, subjects were affected by treatment, but this was not reflected in body mass (the third explanation). Although there is literature suggesting that body mass of nestlings is determined to a large degree by environmental factors (e.g. van Noordwijk et al. 1988; Christie et al. 2000), there are also instances where adult body mass appears to be determined to a great degree by genetics. One such example is from Schmidt et al. (2012), where adult body mass of song sparrows captured from the wild and hand-raised from PHD 3 was significantly correlated to body mass of their free-living fathers, even though father and offspring presumably did not share similar developmental and adult environments.

Reports of effects of nutritional stress on metabolic rate in birds have been inconsistent. While some researchers have found that poor developmental conditions elevate metabolic rates (e.g. Verhulst et al. 2006; Criscuolo et al. 2008; Schmidt et al. 2012), other researchers, including ourselves, have found no effect (e.g. Krause et al. 2009). These inconsistencies may be dependent on species and type of developmental stress employed. For example, Verhulst et al. (2006) manipulated brood sizes of zebra finches, Criscuolo et al. (2008) manipulated dietary protein intake of zebra finches, and Schmidt et al. (2012) manipulated quantity of food intake of song sparrows. Krause et al. (2009), who used similar manipulations as Criscuolo et al. (2008) but found no effect of their treatment on resting metabolic rate in zebra finches suggest that effects of early nutritional treatment may resurface under stressful conditions in adulthood. Thus, in addition to any variation introduced by different treatment methods, measures of metabolic rate may also be sensitive to current nutritional status.

In conclusion, the results from the present study provide support for phenotypic programming, where an interaction of early and late developmental conditions influences multiple aspects of adult physiology. Previous research has suggested that very early life conditions can shape adult phenotypes, and that in addition to environmental quality, environmental instability may be an important factor in determining fitness of the adult organism (Hales and Ozanne 2003; Wells 2007; Monaghan 2008). Our findings add to this growing body of research by suggesting that phenotypic programming can also occur in later

stages of development, hence this factor should be taken into consideration in future experiments.

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CHAPTER 3

NUTRITIONAL STRESS AT DIFFERENT DEVELOPMENTAL PERIODS: EFFECTS ON ASSOCIATIVE LEARNING, SPATIAL MEMORY, AND NEOPHOBIA IN ZEBRA FINCHES (TAENIOPYGIA GUTTATA)

Buddhamas Kriengwatana, Sean D.T. Aitken, Laura Garcia, Tara M. Farrell, & Scott A. MacDougall-Shackleton

3.1 ABSTRACT

Developmental environments can have long-term effects on learning and cognition. Multiple aspects of cognition may be affected by unfavorable conditions during development if underlying systems are maturing simultaneously. We investigated the effects of nutritional stress at different stages of development on adult behavioral flexibility, spatial memory, and neophobia in a songbird. Zebra finches (*Taeniopygia guttata*) were raised in consistently high (HH) or low (LL) food conditions until 65 days post-hatch (DPH), or were switched from high to low conditions (HL) or vice versa (LH) at 35 DPH. Subjects were then tested as adults with two associative learning tasks (color and spatial), a spatial food-finding task, and a test of neophobia. The HL treatment significantly impaired birds' ability to learn the spatial association task, and particularly affected females' ability to inhibit responding to the previously learned color stimuli during spatial task trials. Both the HL and LL treatments impaired performance on the spatial memory task, although LL treatment affected both sexes equally while HL treatment had a stronger effect on males. There was no effect of treatment on neophobia. These findings emphasize the importance of both sex and timing for the effects of developmental stress on adult cognitive and behavioral outcomes.

Keywords: developmental stress, learning, cognition, spatial memory, avian, perseveration

3.2 INTRODUCTION

Suboptimal developmental conditions have been shown to produce long-lasting influences on adult phenotypes (Lindström 1999; Tschirren et al. 2009; Boogert et al. 2011). In songbirds, developmental conditions can affect various aspects of cognition such as song learning (e.g. Buchanan et al. 2004; Spencer et al. 2003), spatial memory (Farrell et al. 2012; Pravosudov et al. 2005), and associative rule-learning (Fisher et al. 2006). Moreover, some of these traits are correlated with each other. For instance, song quality positively predicts spatial memory (Farrell et al. 2012) and associative learning (Boogert et al. 2008). Keagy et al. (2012) also recently reported that male display quality, a measure of global cognitive function, and mating success were significantly correlated in the satin bowerbird (*Ptilonorhynchus violaceus*). If the quality of one trait is reliably associated with the quality of other traits, an individual may be able to perform a rapid assessment of one quality and generalize this information to other aspects of condition, which would be advantageous in situations such as female mating decisions (Spencer & MacDougall-Shackleton 2011; Boogert et al. 2011).

Developmental stress may affect quality of multiple functionally independent traits in adulthood if the development of those traits has similar windows of sensitivity to environmental influences (Spencer and MacDougall-Shackleton 2011). Of central importance to this hypothesis is the consideration of the timing of events, as aversive or stressful events may selectively affect only the traits that are developing at the time the event is experienced. The effects of stress on affected traits could manifest immediately or reappear later in life. Western scrub jays (*Aphelocoma californica)* exposed to stress from early post-hatching until nutritional independence had reduction in the size of hippocampus as adults (Pravosudov et al 2005) and similarly manipulated zebra finches (*Taeniopygia guttata*) and song sparrows (*Melospiza melodia*) had reduction in the size of the nucleus HVC (proper name) of the song control system as adults and as juveniles, respectively (Buchanan et al. 2004; MacDonald et al. 2006). The relationship between adult song complexity and spatial memory (Farrell et al. 2012) may thus be due to an overlap of the neural developmental schedules of the hippocampus and the song-control system. In zebra finches HVC develops from approximately post-hatch-day (PHD) 10 to 50 (Bottjer et al. 1985; Nordeen & Nordeen 1988), and although the development timeline of songbird hippocampus in birds is poorly

understood it similarly appears to extend well past the early nestling stage (Clayton 1992; Clayton & Krebs 1994).

Despite the potential effect of timing of stress on development and function of various traits, few studies on cognition in birds have taken this factor into consideration. A recent study on Japanese quail demonstrated that prenatal and postnatal stress had contrasting behavioral outcomes in adulthood (Boogert et al. 2013). The timing of stress in postnatal development has also been shown to have effects on learning and behavior. Zebra finches that exhibited the greatest catch-up growth (rapid increase in body mass compared to normally developing conspecifics) due to poor diets before nutritional independence were less exploratory (Krause & Naguib 2011) and slower to learn an associative learning task (Fisher et al. 2006). Given these findings, traits (such as spatial memory) that previous studies have shown were impaired by adverse developmental conditions (Farrell et al. 2012; Pravosudov et al. 2005) may be differentially affected depending on the time the poor conditions are experienced.

In the current study, we test the hypothesis that the effects of stressful nutritional conditions during development on adult cognition and behavior depend on the developmental period that stress is experienced. Throughout this paper we use the term juvenile to refer specifically to birds that are independent of parental care but are still sexually immature. We manipulated diets of zebra finches during early and/or juvenile development and tested spatial memory, associative learning, and neophobia of these birds as adults. Our results suggest that persistent nutritional stress leads to poorer hippocampus-dependent spatial memory, but switching to nutritionally stressful conditions during later development impairs spatial associative learning.

3.3 METHODS

3.3.1   Animals and Manipulation

Birds used in this experiment were the same birds as chapter 2. Briefly, we manipulated food accessibility during two different phases: the early phase (PHD 6 – 34), and the juvenile phase (PHD 35 – 61). There were two feeding treatments: birds in the high feeding treatment (H) were given access to 65g seed and 13.5g egg-food daily, while birds in the low feeding treatment (L) were given access to 50g total of seed in a mixture containing a 1:3 ratio of seeds and woodchips (by volume), and 6.5g egg-food daily. This manipulation has been used

by many others and has been shown to negatively affect body mass, adult song control brain regions, and song characteristics of zebra finches (Lemon 1993; Spencer et al. 2003; Buchanan et al. 2004; Zann & Cash 2008). We raised offspring zebra finches on H or L feeding treatment during the early phase and then switched feeding treatments for half of the birds in each of the H and L groups during the juvenile phase. This resulted in four feeding treatments: HH, HL, LH and LL. After the juvenile phase, all birds were given *ad libitum* seed. Offspring were kept with their parents until PHD 90 to ensure that young males learned song from their fathers exclusively (Adret 1993; song data were collected for chapter 5) and then housed in same-sex groups of four to five birds. All birds were at least 100 days of age before being subjected to any of the behavioral tests.

3.3.2 Associative learning tasks

Birds were tested on these associative learning tasks when they were approximately PHD 290 (mean ± SD = 286.47 ± 86.29). Birds first completed shaping trials to teach them to search for food hidden behind pieces of knotted yarn on a wooden block (figure 3.1). After shaping, birds were tested on a color association task and then a spatial association task by an experimenter blind to treatment conditions. Birds had access to water and grit *ad libitum* and were given approximately 4 g seed daily (Living World premium finch seed) at the end of every shaping and testing session. The daily requirement for a captive zebra finch is about 3 g of seed (Zann 1996) We determined in a pilot study that this feeding protocol kept birds motivated to complete the task while maintaining approximately the same mass as their free-feeding mass.

Shaping: For stage 1 of shaping, birds were moved into individual cages (36 × 25 × 30cm LWH) and their food cups were removed. A 1 cm thick wooden block measuring 12.5 × 7.5 cm with 12 holes (two horizontal rows of 6 holes) was placed flat on the cage floor. Each hole was approximately 0.5 cm deep and holes were spaced 1.5 cm apart. Six red and six orange pieces of knotted yarn were taped to the block to partially cover the holes, in which 5 g of seed was placed. This was to familiarize birds with experimental apparatus and eliminate effects of neophobia during testing.
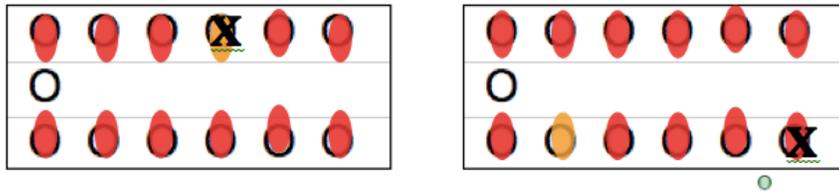
Color task   **Spatial task**

Figure 3.1 Graphic representation of the apparatus for associative learning tasks

Birds had to learn to look for food behind the pieces of red or orange yarn using a color or spatial association rule. The hole in the center was never rewarded during training or testing. If the "x" indicates presence of the food reward, then the color task required birds to search under the orange yarn (left). The location of the orange yarn was randomized for every trial. The color association task was always learned before the spatial association task. The spatial association task required birds to search in the bottom-right corner (right) and ignore the orange yarn. The location of the orange yarn continued to be randomized for every trial during the spatial association task. Only one hole contained food reward on any trial during testing.

The next day the bird progressed to stage 2. The experimenter refilled all of the holes with seed, covered each hole with a piece of red or orange yarn, and placed the wooden block upright, flush with the back of the cage. Birds were then given a maximum of 3 min to search the block for seeds by pecking at the pieces of yarn to remove them and uncover the contents in the hole. Birds were allowed to search in this manner 5 times, and if on 3 of these trials the subject removed yarn from at least 10 of the 12 holes, it continued onto stage 3 of shaping. If it failed, stage 2 was repeated the following days until the criterion was met. The procedure and criterion for stage 2 was repeated on shaping stage 3, 4, and 5, with the only difference being that food was only available in 6, 3, and 1 of the holes, respectively. Once birds completed stage 5, at which point they had learned to search the block until they found the single hole that contained seed, testing began the next day.

Color association task: In the color association task subjects had to learn that the color of yarn (red or orange) indicated the food reward. On each trial, the food reward was available in only one of the holes, which was always covered by the target color yarn. The target color was randomly assigned to each bird, and there was only one piece of yarn of the target color on the block on any trial (e.g., 11 red distractors and 1 orange target). Subjects were given 32 trials a day, and the criterion for learning was that on five consecutive trials they obtained the food reward by choosing the target color yarn as their first choice.

Spatial association task: The spatial association task began the day after birds learned the color association task. The arrangement and procedure for the spatial association task was exactly the same as the color association task, except that the target color yarn no longer predicted the food reward; instead the food reward was always located in a hole in one of the four corners of the block. The position (i.e. top left, top right, bottom left, bottom right) of the correct location was randomized across subjects, but remained consistent for individuals. The location of color yarn was pseudo-randomly assigned on each trial, such that it was never on the hole where the birds were rewarded for the spatial association task. This was because during a pilot study (unpublished results) we found that birds had great difficulty learning the spatial task if reinforcement of the contingencies used in color association task continued. Birds were given up to 32 trials a day, and the criterion for learning was that on five consecutive trials, they obtained the food reward by choosing the correct spatial location as their first choice. Testing was terminated immediately after birds reached criterion

(regardless of whether they had completed the 32 trials for that day) and birds were returned to their group-housed cages.

We assessed the degree to which birds continued to use the color association task strategy during the spatial task. Perseverative errors may indicate the degree to which an individual is able to inhibit interference from previously rewarded stimuli and acquire a new learning rule (Ragozzino 2002). Spatial association task trials were grouped into blocks of four, and a perseverative error was scored if the bird chose the target color yarn first on at least 50% of the trials in a block (i.e. at least 2 trials in a block of 4). Thus, the maximum number of perseverative errors a bird could make per day was eight. Trials that did not form a complete block were not counted towards perseverative errors. For example, if a bird reached criterion at 22 trials, then only the first 20 trials were scored for perseverative errors. Ragozzino (2002) and Floresco et al. (2006) have used a similar but slightly more conservative method to score perseverative errors (i.e. at least 3 trials in a block of 4) in tasks that measured behavioral flexibility in rats. However, their task was conducted on a T-maze and thus there were only two choices on any given trial, while in our task there were twelve choices.

3.3.3   *Hippocampus-dependent spatial memory task*

Birds were tested on the spatial memory task when they were approximately PHD 480 (mean ± SD = 482.57 ± 44.71). This task was similar to the hippocampus-dependent spatial task used by Bailey et al. (2009). The task consisted of (1) a training phase where birds learned the location of food, and (2) a testing phase where birds underwent four probe trials to determine the accuracy of their spatial memory. Training and testing concluded on the same day (details below). We conducted training and testing in a separate room that contained the experimental cage, measuring $95 \times 52 \times 92$ cm (LWH) and elevated 75 cm above the floor, with two entrances on opposite sides of the cage (figure 3.2). The orientation of the cage within the room was consistent throughout the entire experiment and there were prominent visual cues within the room (e.g. shelving units, table, brightly-colored door). Two vertical dividers each with an opening in the center partitioned the experimental cage into three approximately equal-sized compartments, so that subjects were able to see only the food cups of the compartment they were currently in, but were also able to freely visit the other two compartments. The left and right compartments each contained four identical food cups that remained in the same positions throughout the experiment, whereas the central compartment
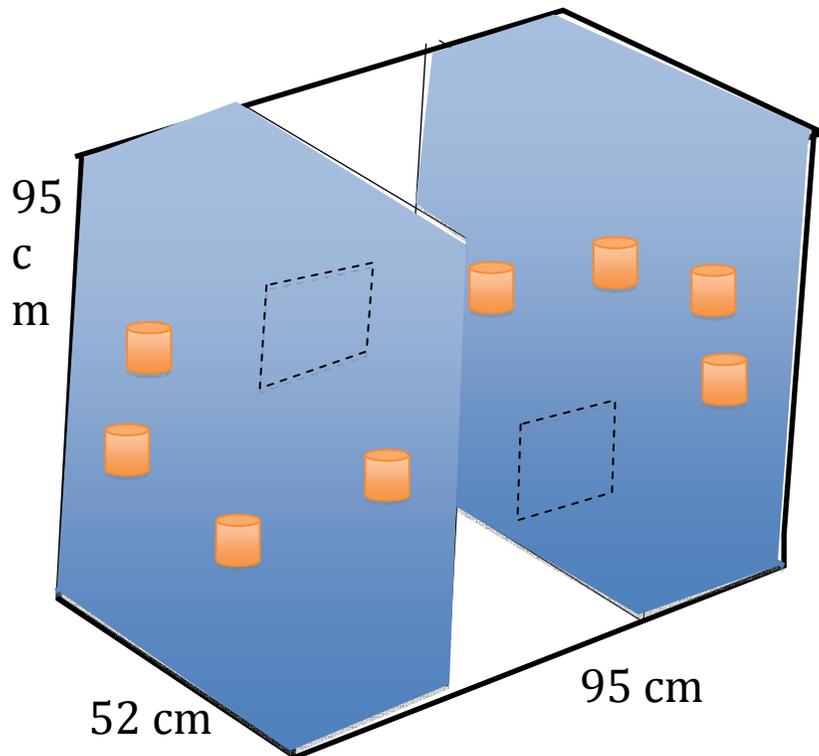
Figure 3.2 Graphic representation of the apparatus for the hippocampus-dependent spatial memory task

The apparatus was a large cage divided into three compartments. Birds entered into the central chamber from one of two entrances (squares with dotted lines). During training trials birds always entered from one of the two entrances. The two compartments on the side each contained four food cups. The position of the cups was the same during the entire experiment. For any individual, only one of the cups was baited with food, and this cup was the same throughout testing and training. In training trials, birds had to remember the location of the baited cup. Once birds remembered, they were removed from the apparatus for 1 h. After this 1 h retention interval, four probe trials were given to assess spatial memory. One these probe trials, birds had to locate the baited cup after entering from the entrance they had used during training, as well as after from the entrance they had never used before. Small woodchips were placed in all the cups during probe trials to prevent birds from being able to see the seeds.

remained empty. Birds were released into the central compartment from one of the two entrances at the start of each trial. A video camera was positioned in the room so that an experimenter could observe the bird's movements in the cage from an adjacent room. The experimenter was blind to treatment conditions.

On the day before training and testing, the focal bird was moved to a holding cage ($36 \times 25 \times 30$cm LWH) with food, water, and grit ad libitum. The next day the focal bird was food deprived for 4-5 h and then given 1 h to freely explore the experimental cage with a companion bird. Birds were more likely to explore all three compartments of the experimental cage if there was another bird present (personal observation). Immediately before training, both birds were removed from the experimental cage and one of the cups in the experimental cage was baited with one teaspoon of seed. This same cup was baited for all training and testing trials for an individual bird, but the location of the baited cup was randomized across birds. The focal bird then entered the experimental cage alone and was given 30 min to locate the baited cup. In training trials, the focal bird always entered from the same entrance. Birds were allowed to eat from the baited cup for 30 sec before they were put back into their holding cage for 5 min. For the rest of the training trials, birds were only given 15 min per trial to search. If the bird visited the baited cup first within 30 sec after being released into the experimental cage three times consecutively, it was considered to have completed the training phase and was placed back into its holding cage in preparation for the testing phase.

The testing phase began one hour after the bird completed training. The testing phase consisted of four probe trials, which were identical to the training trials except that birds entered the cage from either entrance and inedible wooden chips were added to all cups including the baited cup, to prevent birds from being able to see the seeds. The entrance from which birds entered the experimental cage was randomized across each probe trial. For each probe trial we recorded the number of cups the bird visited before visiting the baited cup, the number of visits the bird made to previously searched cups (revisits), and the time it took for birds to complete the trial. After completion of the four probe trials, birds were returned to their group-housed cages.

3.3.4   Neophobia

This task tested willingness to approach a novel non-food item. Birds were on average PHD 220 (mean ± SD = 220.33 ± 14.87) at time of testing. Birds were first moved to individual cages (36 × 25 × 30cm LWH). Each cage had two perches, one on the left and one on right side of the cage. Food and water were available *ad libitum* throughout the task, and the food cup was placed in the center of the cage to reduce side bias. The task consisted of a 10 min baseline trial and a 10 min test trial, with the order of the trials randomized across birds. A digital video recorder was used to record birds' behavior during baseline and test trials. In the test trial an unfamiliar object (plastic butterfly-shaped hairclip) was attached to one of the perches. For baseline and test trials, an experimenter blind to treatment conditions coded the amount of time birds spent in the half of the cage that contained (or would contain) the unfamiliar object and amount of time birds spent on the same perch on which the object was (or would be) attached to. Which trial birds received first (baseline or test) was randomized to reduce any effects of trial order.

3.3.5   Statistics

Statistical analyses were conducted using SPSS 20.0. We used linear mixed models with restricted maximum likelihood (REML) to determine the effect of treatment on all our dependent measures (i.e., color and spatial associative learning, perseverative errors, hippocampus-dependent spatial memory, and neophobia). This analysis is appropriate for our data because we can control for the potential nonindependence of our samples (i.e. relatedness of siblings in each nest) to avoid pseudoreplication. In all of the analyses described below we first tested the significance of the random effects of broods and individuals nested in broods by using maximum likelihood theory to compare fitting of data into a similar model without the random effect. As these random effects did not contribute significantly to all models, some results are reported with these random effects excluded from the final model. All two-way and higher-order interactions were included in the full model and stepwise deletion of non-significant terms was applied to obtain the most parsimonious model of the data. Multiple comparisons were adjusted using Sidak corrections.

Some subjects died before completing testing or were unfit for testing (e.g. hip problems), so the sample sizes for the different tests varied: 30 for the associative learning, 28 for spatial memory task, and 33 for neophobia. Table 3.1 summarizes the dependent variables, fixed and random effects entered into the linear mixed models for measures of associative learning, spatial memory, and neophobia. We did not include age at time of testing as a covariate for neophobia because observations were conducted within three days.

3.4 RESULTS

3.4.1 Associative learning

Nutritional manipulations significantly affected associative learning, but in a task- and sex-specific manner. The number of trials to criterion (TTC) was significantly affected by task ($F(1, 50) = 5.76$, p = 0.020) and feeding treatment × task interaction ($F(3, 50) = 3.20$, p =0.031), but not by feeding treatment ($F(3, 50) = 2.62$, $p = 0.061$). Age also covaried significantly with TTC ($F(1, 50) = 11.22$, $p =0.002$), whereby older birds learned the tasks faster ($t(50) = -3.35$, $p = 0.002$). Pairwise comparisons of the main effect of task indicated that birds found it harder to learn the spatial task compared to the color task ($p = 0.020$; data not shown). Pairwise comparisons of the feeding treatment × task interaction indicated that birds that experienced HL conditions required significantly more TTC than birds that experienced LH conditions ($p = 0.040$; figure 3.3a). Feeding treatment did not significantly affect ability to learn the color task.

One bird in the HL group appeared to be an outlier because its performance on the spatial association task was more than 2 standard deviations above the mean. Exclusion of this bird in a different linear mixed model still revealed a significant effect of task ($F(1, 48) = 6.51$, $p = 0.014$), but the interaction of feeding treatment × task became non-significant ($F(1, 48) = 2.67$, $p = 0.058$). However, pairwise comparisons of the interaction still indicated that HL females required significantly more TTC on the spatial task than LH females ($p = 0.015$). Once again, males were not significantly affected by feeding treatment. Therefore, our results indicate the HL feeding treatment significantly impaired females' ability to learn the spatial association task after first learning the color association task.

Table 3.1. Summary of fixed and random effects of the linear mixed models for analysis of associative learning, spatial memory, and neophobia

Significant random effects that were included in the final model are marked with an asterisk (*). The term individuals nested in broods is abbreviated as indiv(brood). Fixed covariates are italicized.

	DV	Fixed effects	Random effect
Associative learning	# trials to criterion (TTC)	feeding treatment task sex *age*	brood indiv(brood) target color target location
	# perseverative errors	feeding treatment sex *age*	brood indiv(brood)
Spatial memory	# cups searched	feeding treatment sex probe trial # *age*[1]	brood indiv(brood) entry door food location
	# revisits	feeding treatment sex probe trial # *age*[1]	brood indiv(brood)* entry door food location
	time to complete trial (s)	feeding treatment sex probe trial # *age*[1]	brood indiv(brood)* entry door food location
Neophobia	time (s) spent in half of cage with object	feeding treatment sex condition type (baseline/test)	brood indiv(brood) condition order
	time (s) spent on perch with object	feeding treatment sex condition type (baseline/test)	brood indiv(brood) condition order

[1] Age was non-significant and removed from the final model.

a

b
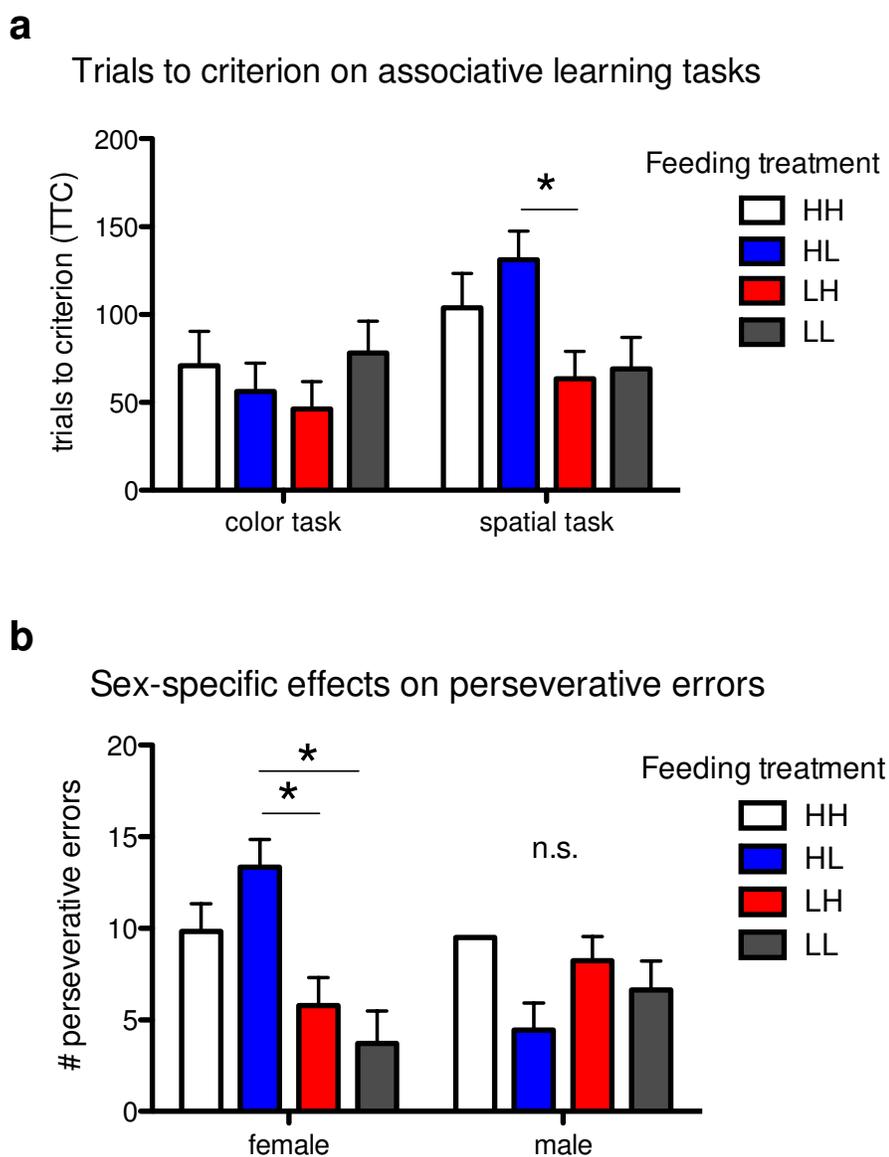
Figure 3.3. Interaction of feeding treatment × task for trials to criterion (TTC) on associative learning tasks and feeding treatment × sex for perseverative errors

Associative learning and perseverative errors were significantly affected by feeding treatment. The HL group found it significantly harder to learn the spatial task compared to the LH group (a). Females in the HL group made significantly more perseverative errors than females in the LH and LL group, but males of different groups did not differ on the number of perseverative errors (b). Error bars are ± 1 SEM. No error bars are depicted for HH males in (b) because there was only one bird in this group.

3.4.2 Perseverative errors

Perseverative errors were significantly affected by the interaction of feeding treatment × sex ($F(3, 22) = 3.753$, $p = 0.026$), but not by feeding treatment ($F(3, 22) = 1.90$, p = 0.158) or sex ($F(1, 22) = 1.16$, $p = 0.293$). Age was not a significant covariate and removed from the final model. Pairwise comparisons of the feeding treatment × sex interaction indicate that HL females made more errors than LH and LL females ($p = 0.049$ and 0.010, respectively; figure 3.3b). Number of perseverative errors made by males was not significantly affected by feeding treatment. Removal of the outlier still revealed a significant effect of feeding treatment × sex ($F(3, 21) = 3.13$, $p = 0.047$), where HL females made more errors than LH females ($p = 0.027$), but not LL females ($p = 0.877$).

3.4.3 Hippocampus-dependent spatial memory

Feeding treatment significantly affected performance on the hippocampus-dependent spatial memory task. The number of cups searched during probe trials was significantly affected by probe trial # ($F(3, 96) = 5.54$, $p = 0.002$) and the interaction of feeding treatment × probe trial # ($F(9, 96) = 2.31$, $p = 0.022$), but not by feeding treatment ($F(3, 96) = 1.37$, $p = 0.258$). Pairwise comparisons of the main effect of probe trial indicated that birds had to search more cups to find the baited cup in trial 1 compared to trials 3 and 4 ($p = 0.008$ and 0.002, respectively; figure 3.4a). This mirrored results from a previous study (Bailey et al. 2009) and indicates that the first probe trial following the retention interval is the best test of spatial memory, with re-learning likely influencing performance on probe trials 2, 3, and 4. Post hoc comparisons of the feeding treatment × probe trial interaction indicated that LL birds searched more cups than HH birds on probe trial 1 ($p = 0.039$; figure 3.4b).

The number of revisits subjects made to previously searched cups was significantly affected by probe trial # ($F(3, 92.03) = 6.08$, $p = 0.001$), but no significant main effect of feeding treatment or feeding treatment × probe trial # interaction ($F(3, 4.87) = 1.52$, $p = 0.320$ and $F(9, 92.03) = 1.57$, $p = 0.135$, respectively). Pairwise comparisons of the main effect of probe trial # indicated that subjects revisited previously searched cups significantly more often on trial 1 than any other trial ($p = 0.023$ for trial 2; 0.007 for trial 3; and 0.001 for trial 4; data not shown).

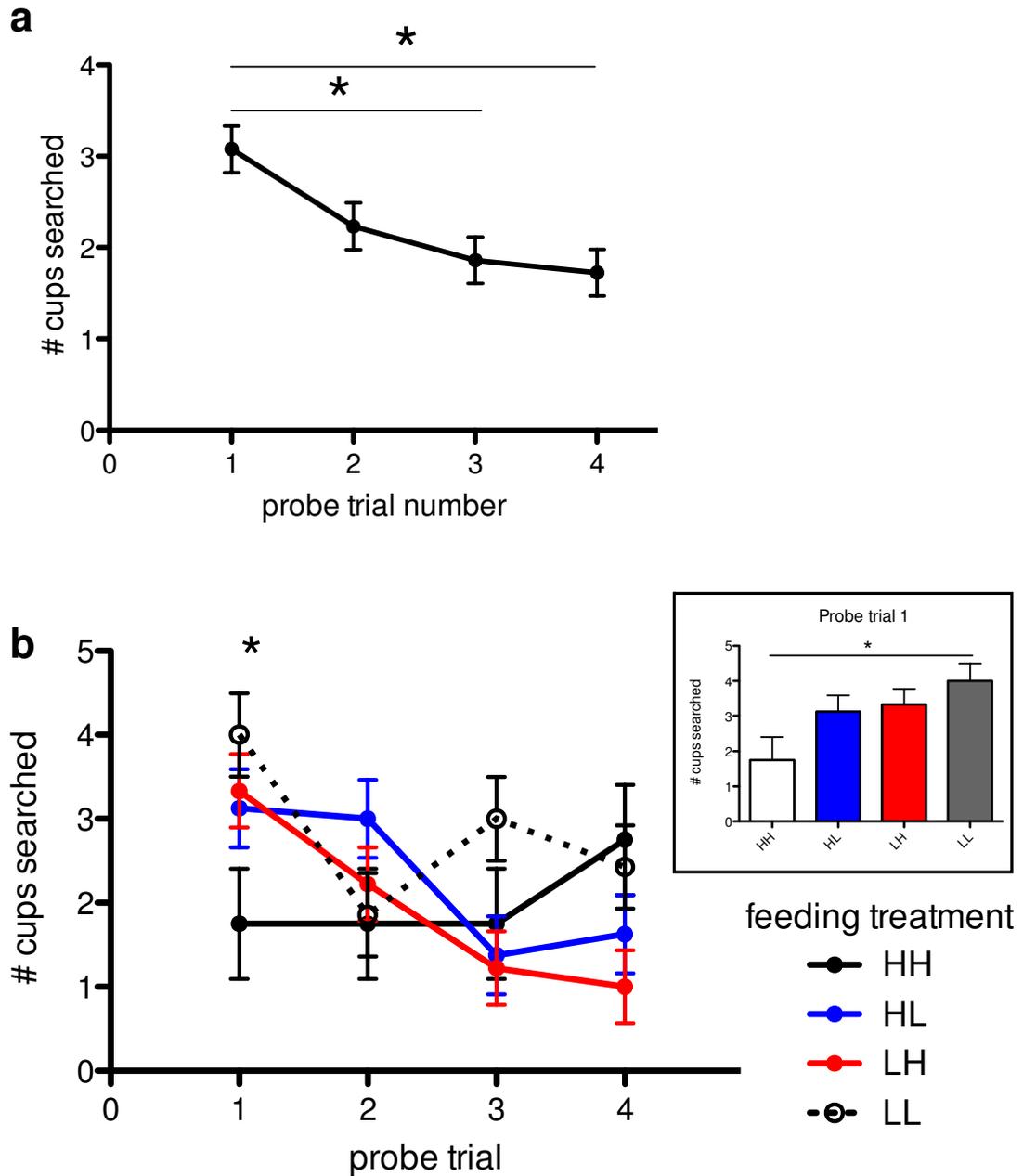**Figure 3.4. Main effect of probe trial # and feeding treatment × probe trial # on number of cups searched in the hippocampus-dependent spatial memory task**
Number of cups searched decreased significantly from probe trial 1 to probe trial 3 and 4, indicating that performance improved over probe trials (a). Feeding treatment impaired spatial memory of the LL compared to the HH group on probe trial 1 (b). Error bars are ± 1 SEM.

The time taken (in seconds) to successfully complete probe trials in the spatial memory task was significantly affected by feeding treatment and probe trial # ($F_{(3, 101)} = 3.59$, $p = 0.016$ and $F_{(3,101)} = 2.83$, $p = 0.042$, respectively), but not by sex ($F_{(1, 101)} = 0.29$, $p = 0.591$). Time taken to complete probe trials was also significantly affected by the interaction of feeding treatment $\times$ sex ($F_{(3, 101)} = 2.77$, $p = 0.045$). The main effects of feeding treatment indicated that HL birds took significantly longer to complete the probe trials compared to HH birds ($p = 0.013$). The main effect of probe trial # indicated that birds took significantly longer to complete probe trial 1 compared to probe trial 4 ($p = 0.026$). Pairwise comparisons of the significant feeding treatment $\times$ sex interaction indicated that the main effect of feeding treatment was driven by males. HL males took significantly longer to complete probe trials compared to HH and LH ($p = 0.032$ and 0.010, respectively; figure 3.5). In females, time taken to complete probe trials was not significantly affected by feeding treatment. These results suggest that HL feeding treatment also impair hippocampus-dependent spatial memory of males, as Bailey et al. (2009) reported that birds with lesions to hippocampus took longer to relocate a baited food cup. Overall, these results suggest that hippocampus-dependent memory is diminished in males in the HL and both males and females in the LL group, but the manifestations of these impairments differ depending on timing of the nutritional manipulation.

3.4.4   Neophobia

Feeding treatment did not significantly affect neophobia. Amount of time (s) spent in the half of the cage that contained the novel object was not significantly affected by feeding treatment or sex ($F_{(3, 28.0)} = 1.14$, $p = 0.349$ and $F_{(1, 28.0)} = 1.91$, $p = 0.178$, respectively). Time spent in the half of the cage with the novel object was almost significantly affected by whether the novel object was present or absent ($F_{(1, 32)} = 4.15$, $P = 0.05$), whereby birds spent less time in the half of the cage if it contained the novel object than in the half of the cage that never contained the novel object (data not shown). Time spent on the perch with the novel object was also not significantly affected by feeding treatment or sex ($F_{(3, 50)} = 1.32$, $p = 0.279$ and $F_{(1, 50)} = 0.88$, $p = 0.352$), but was significantly affected by whether the novel object was present or absent ($F_{(1, 50)} = 59.63$, $p < 0.001$). Birds spent significantly less time on the perch when the object was not present ($p < 0.001$; data not shown).
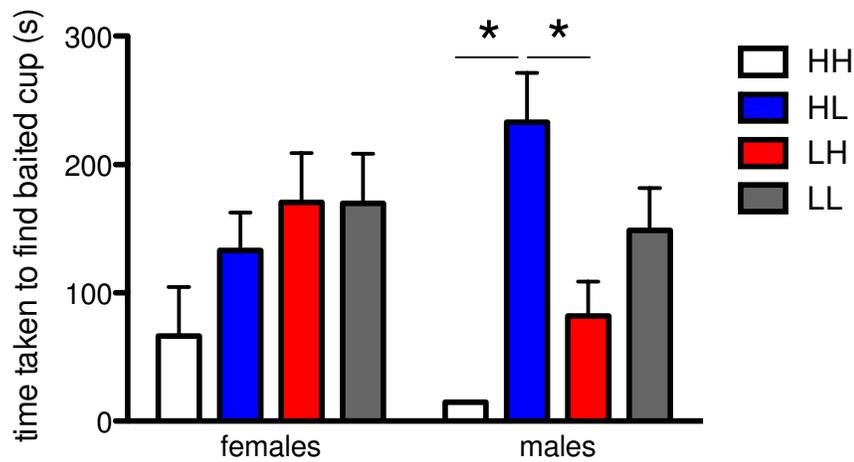
Figure 3.5. Interaction of feeding treatment × sex on time taken to complete probe trials on the hippocampus-dependent spatial memory task

Feeding treatment significantly affected time to complete probe trials for males, as HL males required significantly more time to complete probe trials than HH and LH males. Feeding treatment did not significantly affect the amount of time females needed to complete probe trials. Error bars are ± 1 SEM.

3.5 DISCUSSION

The primary aim of the study was to test the hypothesis that developmental stress could affect traits such as spatial memory, associative learning, and neophobia,in adulthood. The results of the study partially support this hypothesis, as we found that HL treatment impaired performance on the spatial associative learning task and hippocampus-dependent spatial memory. We also found that LL treatment affected hippocampus-dependent spatial memory, although the nature of the impairment may have been different from HL treatment. However, we found no effect of any of our treatments on neophobia.

3.5.1   Spatial memory

Our results indicate that hippocampus-dependent spatial memory can be affected by nutritional stress during development, which is consistent with previous reports of the effect of developmental stress and spatial memory in other species (Pravosudov et al. 2005; Farrell et al. 2012). Our results provide new evidence to suggest that zebra finch spatial memory can be impaired by persistent unfavorable nutritional conditions (LL) or by a switch from favorable to unfavorable conditions during the juvenile period (HL) in males. Birds reared in consistently poor conditions (LL) made more errors on the first probe trial than birds in the control group (HH). Compared to the other probe trials, performance on the first probe trial was not influenced by re-learning, and therefore likely the best test of spatial memory. Males reared in poor juvenile conditions (HL) took more time to find the baited food cup. From these observations, it is possible that the timing of stress may have affected the nature of the effect on hippocampus-dependent spatial memory. Even though HL birds required more time to complete probe trials, their search accuracy remained comparable to controls (HH), whereas LL exhibited the opposite, showing impairments in search accuracy but not search time. A potential explanation for these results is that HL birds formed accurate representations of the correct location but were slower at retrieving the representations. LL birds may not have been slower at retrieving representations, but may have initially formed poor representations of the correct location, leading them to make more erroneous searches.

The diminished performance of birds that experienced L conditions during the juvenile phase also supports work indicating that the avian hippocampus has a protracted development in songbirds (Clayton 1992; Clayton & Krebs 1994). In rats, developmental stress can impair

adult learning and memory on spatial memory related tasks, likely due to its impacts on hippocampal neurons (Isgor et al. 2004; Bedi 2003; Lister et al. 2005; McCormick et al. 1995, 2010; Lemaire et al. 2000). If developmental stress has the same effects on hippocampus in zebra finches, then it is possible that hippocampus-dependent spatial memory of HL and LL groups were affected because hippocampus starts developing early in life, but that the period of greatest sensitivity to environmental input may occur during the juvenile period (Pravosudov et al. 2005; Clayton 1992; Clayton & Krebs 1994).

3.5.2   Associative learning and perseveration

Adolescence may be a stage of high vulnerability to the effects of stress due the extensive degree of brain plasticity exhibited during this age (Romeo 2003; Romeo & McEwen 2006). Our study is the first to suggest a sex-specific effect of stress during the juvenile period on perseveration in birds (having more males in the HH group would strengthen this finding). Recent work by Titulaer et al. (2012) indicated that early rearing conditions did not have any effect on behavioral flexibility in great tits, *Parus Major*, which suggests that effects on perseveration are only evident if stress is experienced as juveniles. However, that the LL group was not similarly impaired, even though they also experienced stress as juveniles indicates that these effects may also depend on a mismatch between early and juvenile rearing conditions. Organisms may use conditions during development as indicators of adult environments and consequently shift developmental trajectories to give rise to an optimal adult phenotype; however changes in environment may cause a mismatch between current and future conditions, thus resulting in a maladapted phenotype (Monaghan 2008; Frankenhuis & Del Giudice 2011). Work by Fisher et al. (2006) supports this view, as adult associative learning was impaired in zebra finches that exhibited catch-up growth when switched from a low protein to a high protein diet. Altogether, these results emphasize the necessity of taking the developmental stage and sex into consideration when attempting to evaluate the effects of developmental stress on learning and cognition.

Developmental stress may increase perseveration in HL females by altering the impact of estrogens on cognition. Interactions between gonadal and stress hormones commonly occur, and localization of estrogen receptors on the hippocampus suggests that estrogens can modulate learning by modifying the plasticity and activity of this region (McEwen 2002; Markou et al. 2005; Korol 2004; Shughrue & Merchenthaler 2001; Gahr et al. 1987, 1993;

Woolley & McEwen, 1992, 1993). The hippocampus has been proposed to mediate response inhibition and perseveration (Douglas 1967; Gray & McNaughton 1982; Kimble 1968), possibly via effects on locomotor activity (Bast & Feldon 2003). The effects of early life stress on hippocampus have also been shown to be sex-dependent. Hippocampus neurogenesis increased in male, but decreased in female rats almost three weeks after they had experienced 24 h maternal separation (Oomen et al. 2009). If the poor performance on the spatial memory task and greater number perseverative errors exhibited by the HL group can be attributed to deficits in hippocampus function, then this provides additional evidence that hippocampus is particularly sensitive to nutritional stress during the juvenile period because multiple functions of the hippocampus were impaired.

Increased perseveration exhibited by the HL group was concomitant with a significant increase in the number of TTC for the spatial association task, although the effect on TTC was consistent across sexes. Hippocampal lesions can also affect spatial memory and or response inhibition without affecting visual memory or discrimination (Colombo et al. 1997a, 1997b; Alvarez et al 1995; Sherry & Vaccarino 1989; Hampton & Shettleworth 1996), but we cannot discriminate whether the poor performance of the HL group on the spatial association task was due to a spatial memory impairment or response inhibition deficit. Future experiments that counterbalance the order of the spatial and color task would help to disentangle these effects.

In conclusion, our findings indicate that nutritionally stressful conditions during development can have persistent long-term effects on learning and cognition. In particular, our study highlights the importance of considering the impact of stress during the juvenile period on hippocampus-dependent spatial memory, as it seems to be most sensitive to environmental conditions at this time. The sex-specific effect of juvenile stress on males compared to females emphasizes the significant difference between how males and females respond to stress (e.g. McCormick & Matthews 2007), and encourages future investigations of developmental stress and cognition to take sex differences into account.

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CHAPTER 4

NUTRITIONAL STRESS AT DIFFERENT DEVELOPMENTAL PERIODS: EFFECTS ON CATCH-UP GROWTH, ASSOCIATIVE LEARNING, BEHAVIORAL FLEXIBILITY AND BODY FAT OF ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

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4.1 ABSTRACT

Organisms that experience poor followed by rich nutritional conditions during development may display a short burst of accelerated growth (catch-up growth). Catch-up growth is associated with altered adiposity later in life but its effects on cognition are not well defined. Moreover, the effects of catch-up growth are often difficult to distinguish from the effects of poor early life conditions. We test the hypothesis that catch-up growth has unique effects on body fat, associative learning, and behavioral flexibility in a songbird. Zebra finches (*Taeniopygia guttata*) were raised in consistently high (HH) or low (LL) food conditions until post-hatch day (PHD) 62, or were switched from high to low conditions (HL) or vice versa (LH) at PHD 34. Body fat mass was measured throughout development and once in adulthood. Cognitive tests were also administered in adulthood. We found that LH conditions induced catch-up growth, but these conditions also resulted in LH females requiring significantly more learning trials to reach criterion on the associative learning tasks. However we found no effect of early or juvenile nutritional conditions on perseverative errors or body fat mass. Our results point to the possibility that catch-up growth induces a trade-off between growth and cognitive function in females. Either catch-up growth as juveniles does not have long-term effects on fat mass, or it does not have the same programming effects on adiposity in birds compared to mammals.

4.2 INTRODUCTION

Poor nutritional conditions during development can have adverse immediate or delayed, as well as transient or enduring effects on various physiological and cognitive functions (Weaver 2009; Barnes & Ozanne 2011). Shortage of nutrients during early development may impair the growth of particular tissues in order to preserve the growth and function of other tissues necessary for temporary survival, sexual attractiveness, or reproductive capabilities (Hales & Barker 1992; Birkhead et al. 1999; Finch & Kirkwood 2000; Walling et al. 2007). However, if nutritional environments subsequently improve, young organisms reared in resource-poor environments may be able to "catch up" in size relative to normally developing conspecifics by accelerating growth rates for a short period of time (Arendt 1997). This catch-up growth is advantageous if organisms can benefit from being larger – larger individuals are more likely to hold larger territories, win territorial disputes, reach reproductive maturity, survive harsh environments, and have greater mating success (Keeley 2000; Johnsson et al. 1999; Craig & Ragen 1999; Pangle et al. 2004; Choudhury et al. 1996; Sokolovska et al. 2000).

Nevertheless, catch-up growth also incurs significant costs. In humans catch-up growth is associated with increased risk of childhood and adult obesity, insulin resistance and coronary heart disease (reviewed in Ong 2007; Singhal 2010). The effects of catch-up growth on cognition are more ambiguous, with some studies reporting a significant negative association between early postnatal weight gain and adolescent IQ (Estourgie-van Burk et al. 2009), and others reporting a positive association (Lundgren et al. 2001). There have also been studies that have found no differences between adult IQ of infants who underwent catch-up growth and those who did not (Brandt et al. 2003).

Human studies on catch-up growth are often confounded by low birth weight, so whether effects are due to poor early developmental conditions or due to catch-up growth are often difficult to distinguish. Indeed, having to cope with stressful developmental environments (which could manifest as low birth weight) – and not necessarily catch-up growth – may be the cause for detriments observed. Mueller et al. (2010) reported impaired cognitive control in adolescents that experienced caregiver deprivation in early life. Also, Beyerlein et al. (2010) found no association between rapid postnatal weight gain and childhood IQ in infants born not small for gestational age. In light of these observations, it is clear that experimental

studies controlling for the effects of unfavorable developmental environments are needed to investigate the effects of postnatal catch-up growth on cognition.

Experimental studies in songbirds have found that early nutritional stress affects lifespan, growth, morphology, metabolic rates, volume of specific brain regions, and several types of cognitive functions such as song learning and production, and spatial memory (Birkhead et al. 1999; Schmidt et al. 2012; Buchanan et al. 2004; Spencer et al. 2003; Arnold et al. 2007; Farrell et al. 2012; Pravosudov et al. 2005). In these experiments, catch-up growth often occurred when birds in the experimental group were switched to *ad libitum* or high-quality diets after being previously food restricted. However, these studies also do not control for poor early development conditions without catch-up growth, and consequently the observed effects could have resulted directly from early life conditions, indirectly from catch-up growth, or from a combination of the two factors. However, because catch-up growth is defined as accelerated growth following a period of poor nutrition, it is impossible to fully isolate the effects of catch-up growth from the effects of poor nutrition.

Experiments on zebra finches (*Taeniopygia guttata*) have found that individuals exhibiting catch-up growth had altered adult metabolic rates and learning ability when compared to individuals that experienced poor nutrition without catch-up growth (Criscuolo et al. 2008; Fisher et al. 2006). Catch-up growth also appears to have effects on cognition that are not accounted for by early life conditions: greater catch-up growth was associated with poorer associative learning ability and reduced exploratory behavior (Fisher et al. 2006; Krause & Naguib 2011). Combined with the findings by Beyerlein et al. (2010), these studies suggest that catch-up growth may contribute additive effects on cognitive development and function.

For these reasons, in this paper we test the hypothesis that catch-up growth has unique effects on associative learning, behavioral flexibility, and body fat accumulation in zebra finches. The ability to behave flexibly (i.e. to change response patterns in response to environmental cues) is an important skill that complements associative learning, but whether it is also affected by catch-up growth is unknown. We replicated the feeding treatments used in chapters 2 and 3 by manipulating diets of offspring zebra finches during early and/or juvenile development (i.e. before and/or after nutritional independence). (We use the term juvenile to refer to the period where offspring are independent of parents but still sexually immature).

Amount of body fat during treatment and in adulthood was quantified. We also tested associative learning and behavioral flexibility when birds reached adulthood using the same associative learning paradigm. Consequently, we were also able to test whether the results of the associative learning task obtained in chapter 3 were due to deficits in ability to learn spatial associations or ability to switch tasks by including an additional condition whereby some subjects had to first learn a spatial association task, and then a color association task. Our results indicate that catch-up growth impaired associative learning abilities of females, but had no effect on behavioral flexibility or adult fat mass.

4.3 METHODS

4.3.1 Animals and manipulation

Using the same manipulation as described in chapters 2 and 3, we paired 13 male and female zebra finches in January 2012. None of these birds had been used in the previous experiments described in chapters 2 and 3. The pairs successfully reared 58 offspring – originally there were 64 offspring, but six died before treatment ended. Parents in this study had not bred and reared offspring for the previous studies. Only broods with 4 or 5 nestlings at the start of treatment were included in our experiment. Potential non-independence of nest-mates was controlled statistically (see below). Nests were monitored daily for nesting activity.

To summarize the manipulation used in chapters 2 and 3, we manipulated food accessibility during two different phases: the early phase (PHD 5 – 35), and the juvenile phase (PHD 36 – 62). There were two feeding treatments: birds in the high feeding treatment (H) were given access to 65g seed and 13.5g egg-food daily, while broods in the low feeding treatment (L) were given access to 50g total of seed in a mixture containing a 1:3 ratio of seeds and woodchips (by volume), and 6.5g egg-food daily. This manipulation has been used by many others and has been shown to negatively affect body mass, adult song control brain regions, and song characteristics of zebra finches (Lemon 1993; Spencer et al. 2003; Buchanan et al. 2004; Zann & Cash 2008). We raised offspring zebra finches on H or L feeding treatment during the early phase and then switched feeding treatments for half of the birds in each of the H and L groups during the juvenile phase. This resulted in four feeding treatments: HH, HL, LH and LL. After the juvenile phase, all birds were given *ad libitum* seed. Offspring were kept with their parents until PHD 90 to ensure that young males learned song from their

fathers exclusively (Adret 1993; song data was collected for chapter 5) and then housed in same-sex groups of four to five birds. All birds were at least 100 days of age before being subjected to any of the behavioral tests.

4.3.2 Body mass and growth rates

Birds were weighed daily to the nearest 0.1g on an electronic scale throughout the entire duration of treatment (PHD 5 – 62) and once during adulthood (PHD 285.33 ± 40.54 SD). Growth rates (k) were defined as [log mass$_2$ – log mass$_1$]/[t$_2$-t$_1$] (Fisher et al. 2006; Morton et al. 1985). We calculated growth rate constants (k) for the each of the treatment phases (i.e. early and juvenile phase).

4.3.3 Body fat

Body fat was quantified every 10 days throughout treatment (PHD 15, 25, 35, 45, 55) and once during adulthood (PHD 285.33 ± 40.54 SD) using quantitative magnetic resonance (QMR) body composition analysis with an instrument designed for small birds (model MRI-B; Echo Medical Systems, Houston, TX). With QMR, we are able to obtain a more comprehensive measure of body condition, as we can examine both total mass, and percentage of body fat in proportion to total body mass. This QMR analysis has been shown to accurately and precisely detect lean and fat mass in various bird species including zebra finches (Gerson and Gugliemlo 2011; Guglielmo et al. 2011), and here we report the values accurate to 0.01g. The QMR analyzer was calibrated with 5 or 2.1g of canola oil before each measurement. Coefficients of variation for fat and lean mass are 3% and 0.5%, respectively, and relative accuracies are ±11% and ±1%, respectively (Guglielmo et al. 2011). Lean and fat content were adjusted (raw value x 0.94 for fat mass, raw value x 1.021 for lean mass) according to calibration equations for zebra finches (Gerson and Guglielmo 2011; Guglielmo et al. 2011).

4.3.4 Associative learning and behavioral flexibility

Birds were tested at age approximately PHD 200 (mean = 233.96 ± 60.38 SD). Subjects first completed shaping trials to teach them to search for food hidden behind pieces of knotted yarn on a wooden block. After shaping, subjects completed two associative learning tasks (color and spatial). We used very similar shaping, and identical testing procedures as described in chapter 3. However, this study differs from the previous in that the sequence that

subjects completed these two different associative learning tasks was counterbalanced, and pseudo-randomized to ensure that roughly half the subjects in each group learned the color to spatial switch while the other half learned the spatial to color switch. Counterbalancing the order that subjects learned the two tasks allowed us to distinguish between potential effects of treatment on color/visual associative learning or on ability to behave flexibly by switching learning strategies. Once subjects learned the reward contingencies of first task (task 1; criterion described below), they had to learn the reward contingences of the second task the next day (task 2). Subjects were given 32 trials per day, and when they reached criterion for either of the tasks, testing for that day terminated (even if they had not reached the 32 trials that day). Subjects had access to water and grit was *ad libitum* and were given approximately 4 g seed daily (Living World premium finch seed) at the end of every shaping and testing session.

Shaping: For stage 1 of shaping, subjects were moved into individual cages (36 x 25 x 30cm LWH) and their food cups were removed. A 1 cm thick wooden block measuring 12.5 x 7.5 cm with 12 holes (two horizontal rows of 6 holes) was placed flat on the cage floor. Each hole was approximately 0.5 cm deep and holes were spaced 1.5 cm apart. Six red and six orange pieces of knotted yarn were taped to the block to partially cover the holes, in which 5 g of seed was placed. This was to familiarize subjects with experimental apparatus and eliminate effects of neophobia during testing.

The next day the bird progressed to stage 2. An experimenter refilled all of the holes with seed, covered each hole with a piece of red or orange yarn, and placed the wooden block upright, flush with the back of the cage. Birds were then given a maximum of 3 min to search the block for seeds by pecking at the pieces of yarn to remove them and uncover the contents in the hole. Birds were allowed to search in this manner 5 times, and if on 3 of these trials the subject removed yarn from at least 10 of the 12 holes, it continued onto stage 3 of shaping. If it failed, stage 2 was repeated the following days until the criterion was met.

The procedure for stage 2 was repeated on shaping stage 3. However, birds were required to search the block 6 times: for the first three times, food was available in only 6 holes, and for the last three times food was available in only 1 of the holes. . If subjects successfully removed the yarn from the hole containing seed in all 3 of the latter trials (i.e. only one hole

contained seed), we took this as an indication that they had learned to search the block until they found seed. Testing began the next day.

Color association task: In the color association task subjects had to learn that the color of yarn (red or orange) indicated the food reward. On each trial, the food reward was available in only one of the holes, which was always covered by the target color yarn. The target color was randomly assigned to each bird, and there was only one piece of yarn of the target color on the block on any trial (e.g., 11 red distractors and 1 orange target). The location of target color yarn was randomized across trials and across individual subjects. Subjects were given 32 trials a day, and the criterion for learning was that on five consecutive trials they obtained the food reward by choosing the target color yarn as their first choice.

Spatial association task: The arrangement and procedure for the spatial association task was exactly the same as the spatial association task, except that the target color yarn no longer predicted the food reward; instead the food reward was always located in a hole in one of the four corners of the block. The position (i.e. top left, top right, bottom left, bottom right) of the correct location was randomized across subjects, but remained consistent for individuals. Birds were given 32 trials a day, and the criterion for learning was that on five consecutive trials, they obtained the food reward by choosing the correct spatial location as their first choice.

Perseverative errors: Perseverative errors were defined as continued use of the strategy that was rewarded in the first task while learning the second task (even though the strategy was no longer being rewarded during the second task). These errors indicate the degree to which a bird is able to inhibit interference from previously rewarded stimuli and/or acquire a new learning rule. Trials in the second task were grouped into blocks of four, and a perseverative error was scored if the subject used the search strategy of the first task on at least 50% of the trials in a block (i.e. at least 2 trials in a block of 4). Thus, the maximum number of perseverative errors a bird could make per day was eight. Trials that did not form a complete block were not counted towards perseverative errors. For example, if a bird reached criterion at 22 trials, then only the first 20 trials were scored for perseverative errors. Ragozzino (2002) and Floresco et al. (2006) have used a similar but slightly more conservative method to score perseverative errors (i.e. at least 3 trials in a block of 4) in tasks that measured

behavioral flexibility in rats. However, their task was conducted on a T-maze and thus there were only two choices on any given trial, while in our task there were twelve choices.

4.3.5   Statistics

Statistical analyses were conducted using SPSS 20.0. We used linear mixed models with restricted maximum likelihood (REML) to determine the effect of treatment on all our dependent measures (i.e., body mass, growth, body composition, associative learning, perseverative errors). This analysis is appropriate for our data because we can control for the non-independence of our samples (i.e. relatedness of siblings in each nest) to avoid pseudoreplication. In all of the analyses described below we first tested the significance of the random effects of broods and individuals nested in broods by using maximum likelihood theory to compare fitting of data into a similar model without the random effect. As these random effects did not contribute significantly to all models, some results are reported with these random effects excluded from the final model. Stepwise deletion of non-significant terms was applied to obtain the most parsimonious model of the data. Adjustment for multiple comparisons was applied using Sidak corrections.

To analyze effects of treatment on body mass, mass (g) was entered in the linear mixed model as the dependent variable. Feeding treatment, sex, and age (PHD 5, 34, 35, 62, and 285) and all interactions were entered as fixed effects. Body mass at these ages was selected because it reflects mass of birds at the start and end of the early and juvenile phases. The sample size at the start of treatment was 64 birds, but because 6 birds died before the end of treatment, the final sample size was 58 birds (HH = 14, HL = 17, LH = 14, LL = 13). This sample size was also used in analyses of growth rate and body fat.

To analyze effects of treatment on growth, growth rates (k) were first calculated for the early and juvenile phases (PHD 5-34 and PHD 35-62, respectively). Separate linear mixed models were used for each phase. Both models used the growth constant k as the dependent variable, as well as feeding treatment and sex as fixed effects.

To analyze effects on body fat, fat mass (g) was entered into the linear mixed model as the dependent variable. Feeding treatment, sex, age (PHD 15, 25, 35, 45, 55, 285) and all

interactions were entered as fixed effects, with total mass minus fat mass (g) as a fixed effect covariate (Christians 1999).

To analyze effects of treatment on associative learning, number of trials to criterion was entered in the linear mixed model as the dependent variable. Feeding treatment, sex, task type, task order and all interactions were entered as fixed effects, with age at time of testing as a fixed effect covariate. Target color (red or orange) in the visual task and target location in the spatial task were entered as additional random effects. To analyze effects of treatment on ability to switch tasks, number of perseverative errors was entered in a separate linear mixed model as the dependent variable. Feeding treatment, sex, and task 2 type (color or spatial) and all higher-order interactions were entered as fixed effects with age at time of testing as a fixed effect covariate. Target color and target location were also entered as additional random effects. Two subjects died before completing testing, and perseverative errors could not be determined for one other subject due to experimenter error. Thus the final sample size for associative learning and perseverative errors was 56 and 55, respectively.

Table 4.1 summarizes the dependent variables, fixed and random effects of the linear mixed models used for analysis of body mass, growth, body fat, and associative learning.

4.4 RESULTS

Thirteen pairs of zebra finches successfully reared 58 offspring according to our feeding treatments. The sample size for each feeding treatment was: HH = 14 (5 females, 9 males), HL = 17 (9 females, 8 males), LH = 14 (9 females, 5 males), LL = 13 (5 females, 8 males).

4.4.1   Body mass

Nutritional stress during development had significant effects on offspring body mass and growth rates. Figure 4.1 depicts increase in mean offspring mass of the different groups over the treatment period. Analysis of group differences in mass at different ages revealed a significant main effect of feeding treatment ($F(3, 57.87) = 12.90$, $p < 0.001$) and age ($F(4, 219.38) = 969.86$, $p < 0.001$) but not sex. There was also a significant interaction of feeding treatment × age ($F(12, 219.36) = 2.7$, $p < 0.005$). Pairwise comparisons of the significant

Table 4.1. Summary of fixed and random effects of the linear mixed models for analysis of body mass, growth rates, body fat, and associative learning

Significant random effects that were included in the final model are marked with an asterisk (*). The term individuals nested in broods is abbreviated as indiv(brood). Fixed covariates are italicized.

	Dependent variable	Fixed effects	Random effects
Body mass	body mass (g)	feeding treatment sex age[1]	brood indiv(brood)*
Growth rate (early and juvenile phase)[2]	growth constant k	feeding treatment sex	brood indiv(brood)
Body fat	body fat (g)	feeding treatment sex age *total mass minus fat mass*	brood* indiv(brood)
Associative learning	trials to criterion (TTC)	feeding treatment sex task type task order *age[4]*	brood* indiv(brood) target color* spatial location
	# perseverative errors	feeding treatment sex task type	brood* indiv(brood) target color spatial location

[1]Ages entered were PHD 5, 34, 35, 62, and 285. [2]We used separate linear mixed models for growth during the early and juvenile phase. [3]Ages entered were PHD 15, 25, 35, 45, 55, 285. [4]Refers to age at time of testing
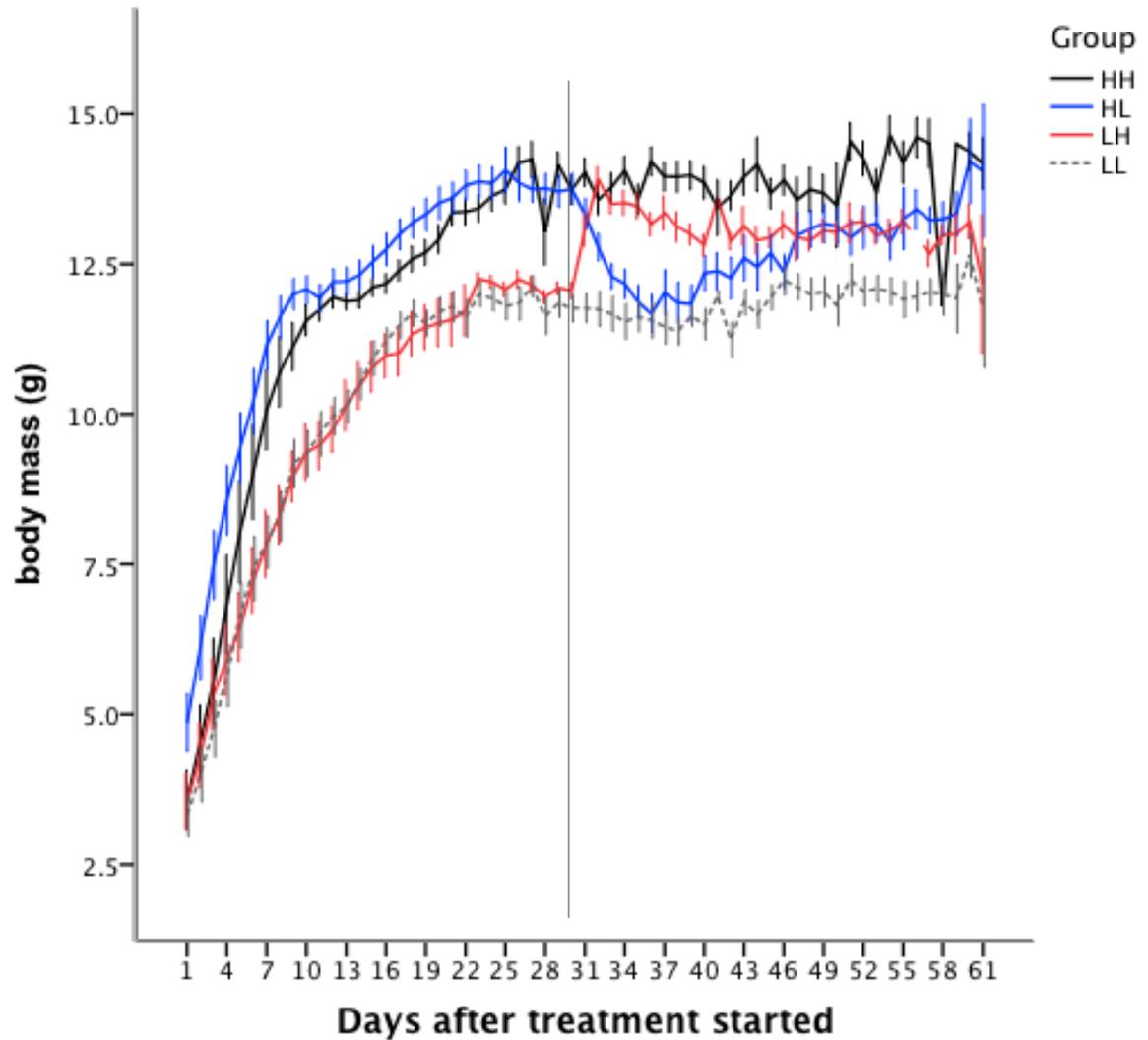
.

Figure 4.1 Increase in mean mass of the different groups over the treatment period

Feeding treatment spanned almost 60 days, with the early phase lasting 30 days (from PHD 5
- PHD 34) and the juvenile phase lasting an additional 29 days (from PHD 35 - PHD 62).
Error bars represent ± 1 SEM. The vertical line denotes the switch from early to juvenile
phase.

main effect of feeding treatment indicated that HH and HL were significantly heavier than LL and LH ($p < 0.05$ for all; figure 4.2a). Pairwise comparisons of the main effect of age indicated that birds were lightest at PHD 5 ($p < 0.001$ for all), heaviest as adults (PHD 285; p<0.001 for all) but that their masses at PHD 34, 35, and 65 did not differ significantly. Post hoc analysis of the feeding treatment × age interaction indicated that during development HH and HL birds were always heavier than LL birds, but that LH birds were able to match HH and HL groups in body mass at PHD 65 and PHD 285 (figure 4.2b). At PHD 34, HH and HL birds were heavier than the LH ($p < 0.01$ and 0.005, respectively) and LL birds ($p < 0.001$ for both), but LH and LL did not differ significantly from each other ($p = 0.946$). The same was true for PHD 35 (HH and HL heavier than LH and LL $p < 0.005$ for all). At PHD 65, HH was significantly heavier than LL ($p < 0.001$), but surprisingly, so was HL (p<0.05). Body mass of LH did not differ significantly from any of the other groups (HH $p = 0.321$, HL $p = 0.998$, LL $p = 0.146$). As adults (PHD 285), HH and HL remained heavier than LL ($p < 0.05$ and 0.005, respectively), but body mass of LH again did not differ significantly from HH ($p = 0.761$, HL $p = 0.073$, or LL $p = 0.544$).

Overall these results indicate that nutritional stress from PHD 5-62 (LL group) had immediate and long-term effects on body mass of zebra finches, but that nutritional stress from PHD 35-62 (HL group) did not. The results also suggest that receiving the H diet from PHD 35-62 after receiving the L diet from PHD 5-34 (LH group) allowed zebra fiches to catch up in body mass after 30 days of H treatment – effects which were not temporary, but persisted into adulthood. Providing *ad libitum* food at PHD 62 after continuous L diet throughout development (LL) did not induce catch-up growth, and birds in this group remained lighter than all the other groups as adults.

4.4.2 Growth

Nutritional stress affected growth rates during the juvenile phase (PHD 35-65), but not the early phase (PHD 5 -34; figure 4.3a). Results of growth rates during the early phase revealed no significant main effect of feeding treatment or sex (raw means and SD for groups HH = 0.0231 ± 0.011, HL=0.0178 ± 0.00782, LH=0.0196 ± 0.00920, LL=0.0194 ± 0.00852). Results of growth rates during the juvenile phase revealed a significant main effect of feeding treatment ($F(3, 50) = 13.90$, $p < 0.001$) but no significant main effect of sex or significant interaction of feeding treatment × sex. Pairwise comparisons of the significant main effect of

a

Main effect of feeding treatment on body mass across all ages
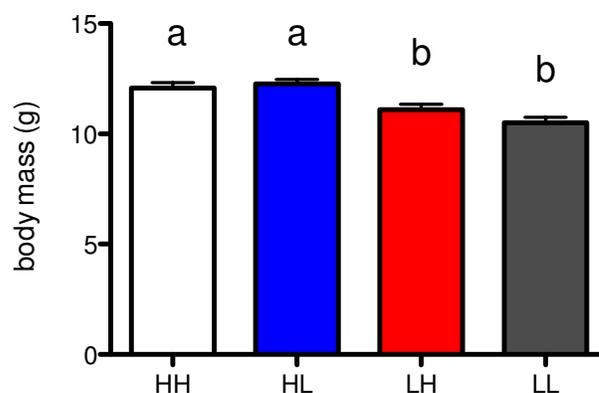
b

Interaction of feeding treatment x age
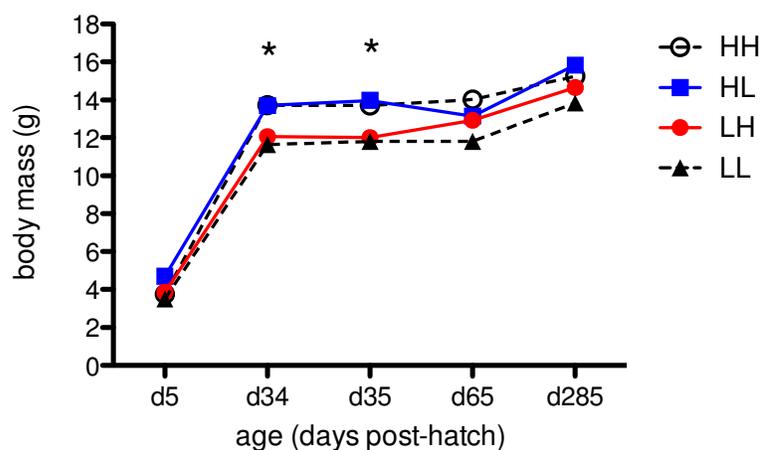
Figure 4.2 Main effect of feeding treatment and interaction of feeding treatment × age on body mass

Body mass was significantly affected by feeding treatment. Letters above data points indicate significant differences (i.e. points that have "a" above them are not significantly different). H feeding treatment during the early phase lead to overall more body mass L feeding treatment during the early phase (a). HH and HL had more body mass than LH immediately before and at treatment switch (PHD 34 and 35, indicated by *), after which LH no longer differed from HH or HL. Error bars are ± 1 SEM. In (b) the error bars are small and hidden behind figure symbols.
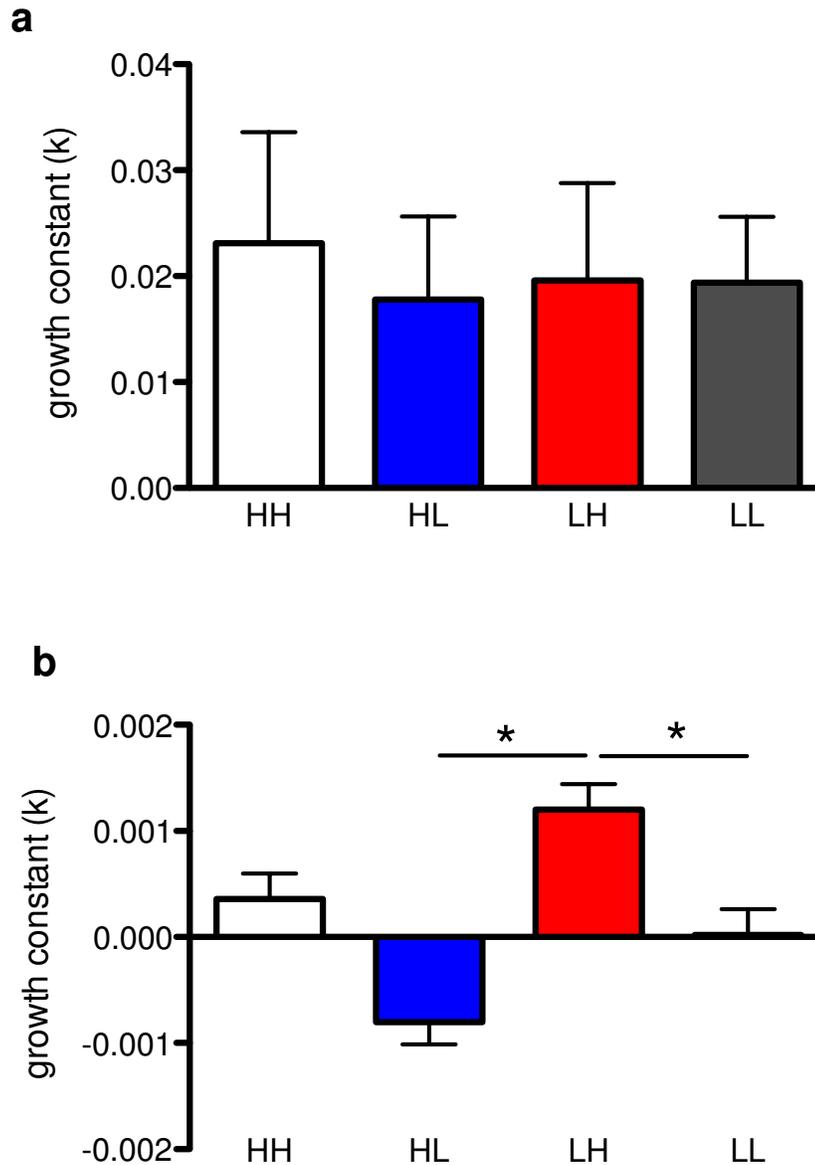
Figure 4.3 Effects of feeding treatment on growth rates early and juvenile growth rates

Feeding treatment did not significantly affect growth rates during the early phase, which ranged from PHD5 – 34 (a). However, LH exhibited faster growth than HL and LL during the juvenile phase (PHD 35 – 62), suggesting that birds in the LH group experienced catch-up growth (b). Error bars are ± 1 SEM.

feeding treatment revealed that HH grew faster than HL (p<0.005), and that LH also grew faster than HL ($p < 0.001$) as well as LL ($p < 0.01$; figure 4.3b).

4.4.3   Body fat

Nutritional stress during development had immediate effects on fat mass, but these effects were not evident in adulthood. Results indicated a significant main effect of age ($F(5,211.83)=24.27$, $p<0.001$), but no main effect of feeding treatment or sex (raw means & SD for groups HH=1.14 ± 0.54; HL=1.28 ± 0.77; LH=1.06 ± 0.50; LL=0.90 ± 0.45). As adults (PHD 285), birds had significantly more fat mass than during development ($p < 0.001$), and birds had more fat mass at PHD 55 than at PHD 35 and 45 ($p < 0.001$ and 0.05, respectively; figure 4.4). There were significant interactions of feeding treatment × age ($F(15,201.99)=2.48$, $p<0.005$) and sex × age ($F(5,204.39)=11.61$, $p<0.001$). Total mass minus fat mass was also a significant covariate ($F(1, 147.10) = 7.71$, $p < 0.01$), whereby birds with more lean mass also had more fat mass ($t(147.10) = 2.78$, $p < 0.01$).

Pairwise comparisons of the feeding treatment × age interaction indicated that treatment affected the ages at which birds had the greatest fat mass, but that fat mass of treatment groups did not differ significantly from each other at any age (figure 4.5a). The HH group had significantly more fat mass at PHD 15 than PHD 35 ($p < 0.05$), and more as adults than during development ($p < 0.005$ for all). The HL group also had significantly more fat mass at PHD 15 than PHD 35 ($p < 0.05$) and more as adults than during PHD 25, 35, and 45 ($p < 0.005$ for all). At PHD 55, HL had significantly more fat mass than at PHD 35 and 45 ($p < 0.001$ for both), and almost had significantly less fat mass than at adulthood ($p = 0.056$). The LH group had significantly more fat mass as adults than during any developmental age ($p < 0.001$ for all). The LL group had significantly more fat as adults than at PHD 35 only ($p < 0.05$). Pairwise comparisons of the sex × age interaction indicated that males and females differed at ages in which they had the greatest fat mass (figure 4.5b). As adults, females had significantly less fat mass than males ($p < 0.001$). During development, females had significantly less fat at PHD 35 than at PHD 55 and 65 ($p < 0.01$ and 0.05, respectively). On the other hand, males had significantly less fat during development than as adults ($p < 0.002$ for all), but fat mass of males during the different developmental ages did not differ significantly.
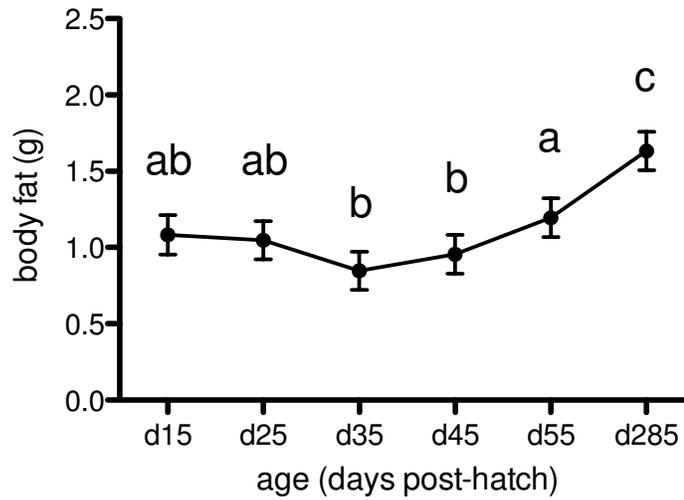
Figure 4.4 Main effect of age on body fat

Amount of body fat varied depending on age. Letters above data points indicate significant differences (i.e. points that have "a" above them are not significantly different). Birds had the lowest amount of body fat at around the time they became nutritionally independent (PHD 30). Birds had significantly more body fat as adults than during development. Error bars are ± 1 SEM.
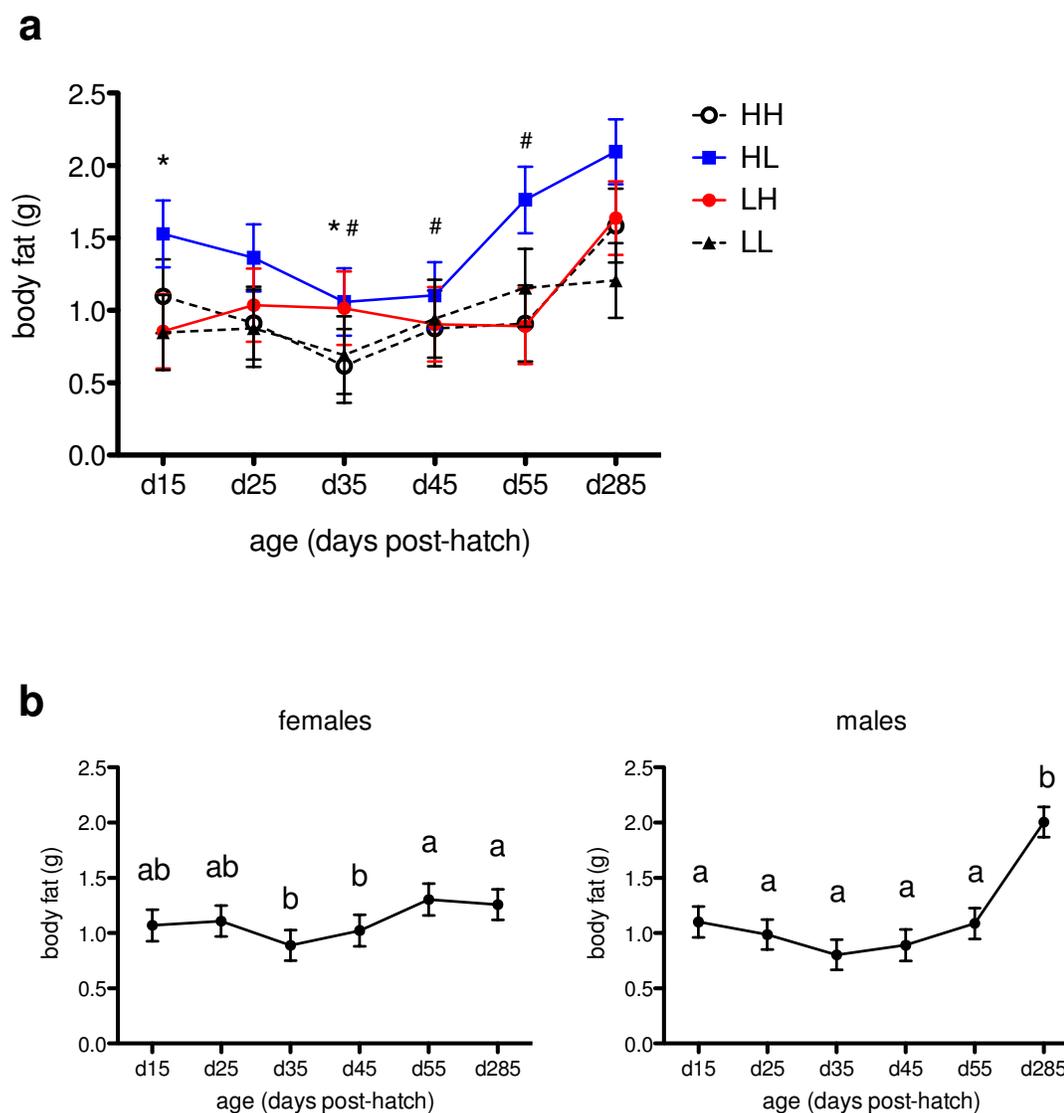
Figure 4.5 Interaction of feeding treatment × age and feeding treatment × sex on body fat

The age at which birds tended to accumulate body fat was significantly affected by feeding treatment. Letters above data points indicate significant differences (i.e. points that have "a" above them are not significantly different). Unlike LL and LH, HH and HL groups had significantly more fat mass at PHD 15 compared to PHD 35 (indicated by *). HL also had more fat mass at PHD 55 compared to PHD 35 and 45 (indicated by #), but this increase did not persist into adulthood (a). Males and females also accumulated fat differently. While both males and females had relatively stable amounts of fat mass during development, only males significantly increased fat mass as adults (b). Error bars are ± 1 SEM.

4.4.4 Associative learning and behavioral flexibility

Nutritional stress during development significantly affected associative learning ability and behavioral flexibility; however these effects depended on sex and timing of stress (figure 4.6). The analysis of nutritional stress treatment on trials to criterion (TTC) indicated that there were no significant main effects of feeding treatment, sex, task type, or task order (raw means and SD for groups HH=64.93 ± 36.00, HL=60.19 ± 40.55, LH=76.04 ±38.58, LL=65.13 ±35.35). However, nutritional stress treatment did significantly affect the two-way interaction of feeding treatment × sex ($F(3, 83.34) = 2.75$, $p < 0.05$), and the three-way interactions of feeding treatment × sex × task type ($F(3, 81.31) = 2.70$, $p < 0.05$), and sex × task type × task order ($F(1, 82.36) = 5.37$, $p < 0.05$). Age was also a significant covariate ($F(1, 83.22) = 19.80$, $p < 0.001$), where older birds required fewer trials to criterion than younger birds ($t(83.22) = -4.45$, $p < 0.001$). Pairwise comparisons performed on the significant feeding treatment × sex interaction indicated the treatment effect was limited to females: LH females required significantly more trials to criterion compared to HH and LL ($p < 0.05$ for both; figure 4.6).

We examined the significant three-way interactions using separate linear mixed models for males and females, and it appears that only females were significantly affected by treatment. Analysis of the feeding treatment × sex × task type interaction revealed that males were not affected – there were no significant main effects of group or task type (raw means & SD for HH=72.06 ± 35.19, HL=56.14 ± 37.95, LH=73.70 ± 32.46, LL=70.75 ± 36.83). Age remained a significant covariate ($F(1, 51.11) = 14.30$, $p < 0.001$), whereby older males required fewer trials than younger males ($t(51.11) = -4.78$, $p < 0.001$). Similarly for females, there was no significant main effect of feeding treatment or task type (raw means & SD for groups HH= 52.10 ± 35.57, HL =63.33 ± 43.28, LH=77.33 ± 42.45, LL = 53.88 ± 31.36). However, there was a significant feeding treatment × task interaction ($F(3, 23) = 3.16$, $p < 0.05$). Pairwise comparisons indicated that the effect was restricted to the HH group, which required significantly fewer trials to learn the spatial task compared to the visual task ($p < 0.05$; data not shown). Analysis of the sex × task type × task order interaction revealed that males were again not significantly affected – there were no significant main effects of task type or task order, or significant interactions of those terms. Age again was a significant covariate ($F(1, 52.22) = 14.95$, $p < 0.001$), whereby older males required fewer trials than
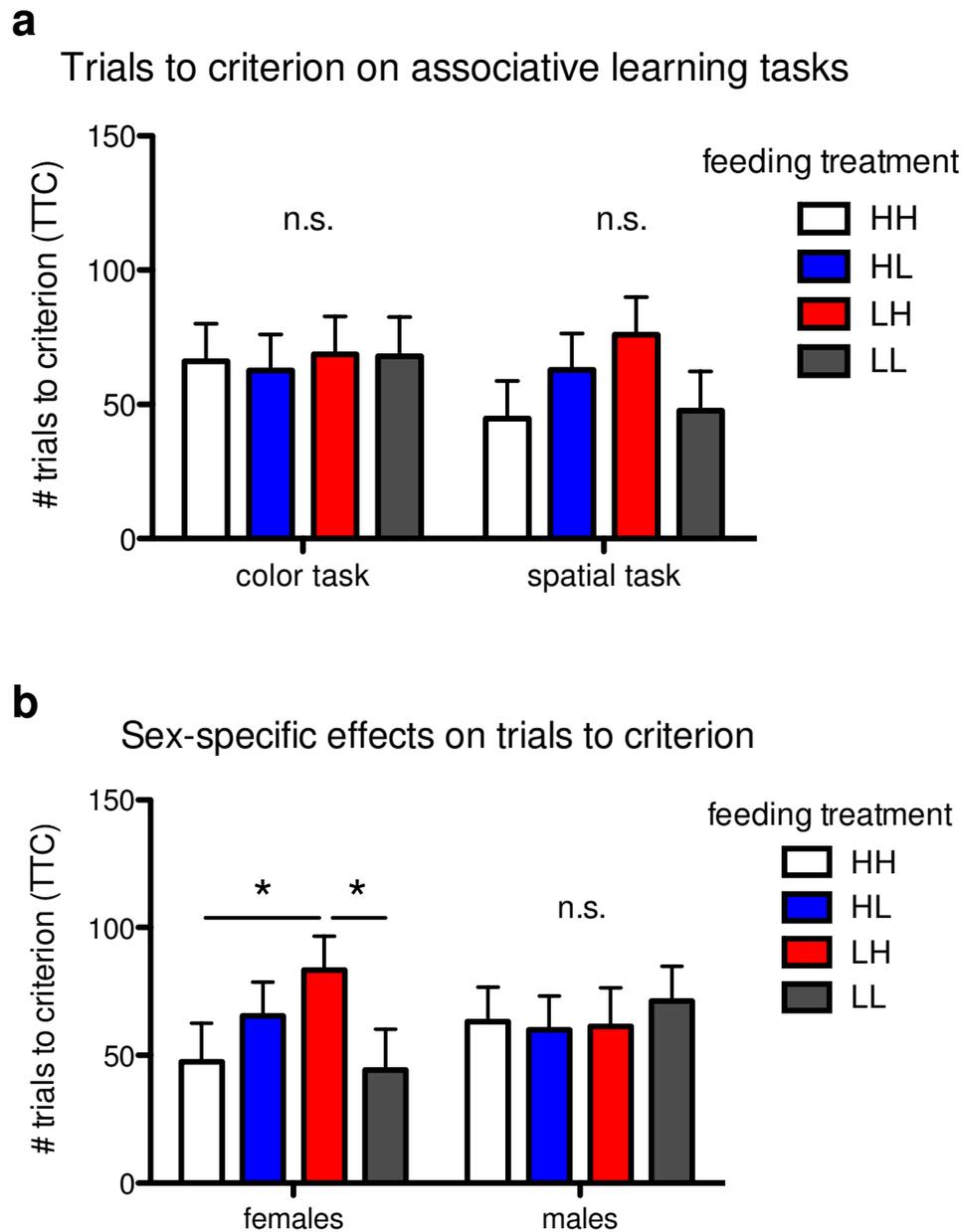
Figure 4.6 Main effect of feeding treatment on trials to criterion (TTC) and interaction of feeding treatment × sex on trials to criterion

There was no significant main effect of feeding treatment on TTC for color or spatial associative learning tasks (a). However, feeding treatment significantly affected associative learning (not specifically color or spatial task) in females only (b). LH females required significantly more TTC than HH and LL females. Error bars are ± 1 SEM.

younger males (t(52.22) = -3.87, $p < 0.001$). For females, there were no significant main effects of task type or task order, however there was a significant interaction of task type x task order (F(1,50) = 5.10, $p < 0.05$). Pairwise comparisons revealed that females required fewer trials to learn the spatial task if it is learned second (i.e. task 2; $p < 0.05$, data not shown).

In summary, associative learning was impaired if females were exposed to unfavorable nutritional conditions early in life followed by favorable nutritional conditions later in life. Learning ability in females was also affected by the type and order of tasks, as they required fewer trials to reach criterion for the spatial task if it was learned second. This suggests that in females, learning a previous (color) association task facilitates learning of the spatial association task, but not vice versa. Males did not appear to be affected by treatment, however, only males demonstrated improved learning ability with increasing age.

4.4.5 Perseverative errors

Nutritional stress during development significantly affected perseverative errors in a sex-dependent manner. Results of the linear mixed model indicated that there were no significant main effects of feeding treatment, sex, or task type (raw means & SD for HH = 2.77 ± 2.56; HL = 2.69 ± 2.80; LH = 4.07 ± 2.59; LL = 3.42 ± 2.61). However, there was a significant interaction of feeding treatment × sex (F(3,33.94) = 3.35 $p<0.05$). Pairwise comparisons of the interaction indicated that LL females made more perseverative errors than LL males ($p<0.05$; figure 4.7).

4.5 DISCUSSION

This aim of this study was to determine whether catch-up growth had effects on body mass, fat mass, and cognitive abilities that were independent of effects of early nutritional stress. Our results indicate that the L during the early phase and H diet during the juvenile phase (LH) induced catch-up growth, such that by the end of the diet manipulation (PHD 62) and as adults (PHD 285), mass of LH birds was indistinguishable from birds that had experienced the H diet throughout (HH, control birds) and from birds that had experienced the opposite switch (HL).
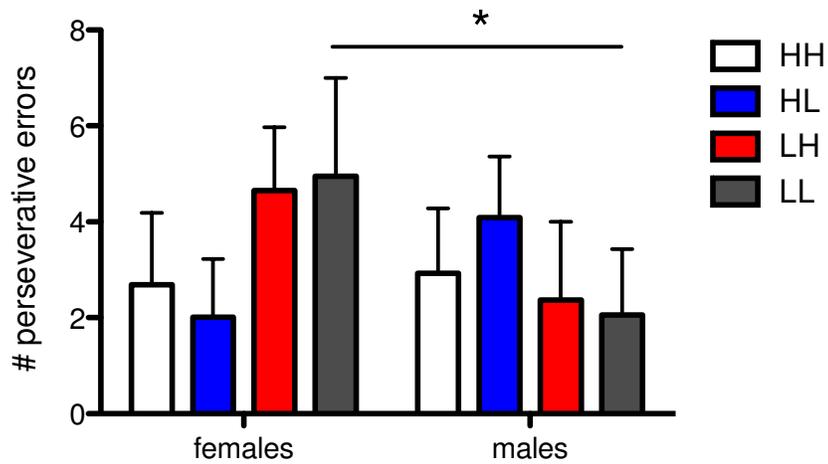
Figure 4.7 Interaction of feeding treatment × sex on perseverative errors

Feeding treatment did significantly affect number of perseverative errors, however LL females made more perseverative errors than LL males. Error bars are ± 1 SEM.

4.5.1   Growth, body mass, and body fat

Consistent with results of chapter 2, we did not observe any effects of nutritional manipulations on growth rates during the early phase (before birds reached nutritional independence). In chapter 2 we suggested that these observations may be due to parents shouldering the costs of stress during this phase in order to buffer effects on nestlings, and consequently offspring did not perceive feeding treatments as stressful. Alternatively, effects of feeding treatment at this phase may not be reflected in body mass. To disentangle these effects, levels of the stress hormone corticosterone of these same birds during the early and juvenile phase were quantified (discussed in chapter 5). Baseline corticosterone was significantly elevated in groups that were experiencing L feeding treatment, suggesting that offspring did in fact perceive the feeding treatment as stressful. These results support our hypothesis that effects of nutritional stress during the early phase may simply not be reflected in body mass.

We also found that neither early nor juvenile nutritional stress (including those that induced catch-up growth) had immediate or long-term effects on fat mass, which is contrary to our predictions. Although in most human studies it is difficult to resolve whether increased adiposity is due to catch-up growth, low birth weight, or the combined effect of these two factors, there are both experimental and observational studies that suggest that catch-up growth during early postnatal development is the principle cause (e.g. Durmu et al. 2010; Howie et al. 2012). Perhaps the reason why we did not observe effects of catch-up growth on fat mass is because we induced catch-up growth in our birds when they were juveniles (around the time of nutritional independence). Consistent with this view, song sparrows (*Melospiza melodia*) treated with the stress hormone corticosterone from PHD 7 - 60 were smaller at PHD 45, but as adult these birds did not differ in adult structural size or fat mass from controls (Schmidt et al. 2012). Alternatively, catch-up growth simply may not have long-term effects on fat mass in birds.

If developmental programming of adiposity is restricted to early life, then nutritional stress at later developmental stages should not have long-term effects on fat mass. This idea is supported by our results of HL treatment. HL birds had managed to regain body mass to match HH birds at PHD 62, likely by increasing stores of fat mass, but this strategy of preferential fat accumulation was only temporary and did not persist into adulthood. Poor

juvenile nutritional conditions in Trinidadian guppies (*Poecilia reticulata*) revealed similar effects (Auer 2010). Even so, in chapter 3, zebra finch males had significantly more fat mass at approximately 2.5 years of age if they experienced L diet compared to H diet during the juvenile period. Therefore, it may be possible that juvenile nutritional stress also affects adiposity, but that these effects appear much later in adulthood.

4.5.2   *Associative learning and behavioral flexibility*

Conditions that induced catch-up growth also reduced associative learning abilities when birds were adults, complementing previous work suggesting that catch-up growth may have long-lasting impairments on cognition (Fisher et al. 2006, but see Bonaparte et al. 2011). However, catch-up growth only affected learning ability of females. The next step would be to determine whether females sacrifice cognitive function for the sake of investing in growth and reproduction (i.e., whether a trade-off exists between catch-up growth and cognitive function in females).

Experiments in chapter 3 also tested associative learning ability of zebra finches in using the switch from color to spatial task. Chapter 3 results indicated that HL conditions impaired learning of the spatial task and caused females in this group to commit significantly more perseverative errors. These results contradict the current findings and those by Titulaer et al. (2012). To examine this discrepancy we determined the *d* effect size (Cohen's *d*) and 95% confidence intervals using raw group means, SD, and Ns for number of trials to criterion and perseverative errors from the two studies (Table 4.2). The effect sizes were large, but the confidence intervals around the effect sizes included 0, suggesting that the differences between these two years were not statistically significant. This suggests that early nutritional stress in females may have large, but highly variable effects, potentially driven by individual differences in the ability to cope with nutritional stressors. Such variation would result in variation across studies in the exact nature of deficits observed.

Neither results from chapter 3, nor results of the current study indicate that catch-up growth affected perseverative errors. This implies that catch-up growth affects only specific cognitive traits. Nevertheless, this study only measured the ability to shift attention between two stimulus modalities (extra-dimensional shifts), but there are many types of behavioral flexibility, such as the ability to shift attention between stimuli of the same modality (intra-

Table 4.2 Comparison of performance on the color (task 1) and spatial (task 2) learning tasks across the two chapters (chapter 3 and 4).

	colour task		spatial task		perseverative errors	
	Cohen's d	**95% CI**	**Cohen's d**	**95% CI**	**Cohen's d**	**95% CI**
HH	0.139	-15.38 – 15.08	1.211	-24.03 –21.35	2.167	-3.99 – -0.17
HL	0.338	-13.55 – 12.83	0.130	-48.71 – 45.76	1.384	-4.74 – 1.70
LH	0.411	-10.41 – 11.29	0.430	-13.20 –12.27	0.966	-2.54 – 0.58
LL	0.557	-13.75 – 14.87	0.377	-15.14 – 15.94	0.134	-1.68 – 1.39

Note: We calculated Cohen's d based on raw means and standard deviations (σ) for each year as: $d = M_1\text{-}M_2/\sigma_{pooled}$ where $\sigma_{pooled} = \sqrt{[(\sigma_1^2 + \sigma_2^2/2)}$

dimensional shifts) and shifting attention to a previously irrelevant cue of the same modality (reversal learning). Thus, it is still possible that catch-up growth and/or developmental stress affected behavioral flexibility, but this study was not able to detect it because we only examined performance on an extra-dimensional shift. An additional note regarding behavioral flexibility is that it has two distinct components: the ability to shift attention between stimuli and the ability to inhibit a previously rewarding response. The paradigm used in this experiment is not able to differentiate between these two components, so adding a test that measures inhibitory control, such as the one used by Boogert et al. (2011), could aid interpretation. Specifically, they used a detour-reaching task that required song sparrows (*Melospiza melodia)* to inhibit pecking impulsively the sides of a transparent cylinder, and instead to go around and enter the cylinder to obtain a food reward.In conclusion, our results reinforce the hypothesis that catch-up growth has negative effects on associative learning ability that are not attributable to merely poor early life nutrition. Males and females may also differ in what costs are paid for catch-up growth, with associative learning ability being one of the trade-offs in females. Our findings also suggest that catch-up growth may only have enduring effects on fat mass if it occurs very early in life, or that increased adiposity caused by catch-up growth does not occur in birds.

ACKNOWLEDGEMENTS

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CHAPTER 5

NUTRITIONAL STRESS DURING DIFFERENT DEVELOPMENTAL PERIODS: EFFECTS ON SONG AND HYPOTHALAMIC-PITUITARY-ADRENAL AXIS OF ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

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5.1 ABSTRACT

In songbirds, developmental stress affects song learning and production. Altered hypothalamic-pituitary-adrenal (HPA) axis function resulting in elevated corticosterone (CORT) may contribute to this effect. We examined whether developmental conditions affected the association between adult song and HPA axis function, and whether nutritional stress at different developmental stages has distinct effects on song learning and/or vocal performance. Zebra finches (*Taeniopygia guttata*) were raised in consistently high (HH) or low (LL) food conditions until post-hatch day (PHD) 62, or were switched from high to low conditions (HL) or vice versa (LH) at PHD 34. Song was recorded in adulthood. We assessed the response of CORT to handling during development and to dexamethasone (DEX) and adrenocorticotropic hormone (ACTH) challenges during adulthood. Song learning and vocal performance was not affected by nutritional stress or catch-up growth. Nutritional stress elevated baseline CORT during development. Nutritional stress also increased rate of CORT secretion in birds that were not stressed before nutritional indpenendence (HL group). As adults, birds in the LL group had lower CORT levels after injection of ACTH compared to the other groups, however there was no effect of nutritional stress on the response to DEX. These results challenge the developmental stress hypothesis and indicate that nutritional stress that affects HPA function may not affect song development in zebra finches.

Keywords: catch-up growth, corticosterone, zebra finch, vocal performance, developmental stress hypothesis

5.2 INTRODUCTION

Growth and developmental processes are sensitive to environmental factors, and early life adversity can have profound effects on behavior and physiology of animals (Lindström 1999; Barnes & Ozanne 2011). Birdsong is a trait that appears particularly sensitive to stressful developmental conditions (Spencer & MacDougall-Shackleton 2011), which indicates that it may be a reliable indicator of developmental history and is one reason why birdsong is a sexually selected trait (Nowicki et al. 1998, 2002). For songbirds such as the zebra finch (*Taeniopygia guttata*), development of a specific network of interconnected brain nuclei mediates song learning and production. Development of these brain regions and song learning behaviors begin soon after hatching and conclude at sexual maturity (Brainard & Doupe 2002; Kirn 2010) Because zebra finches do not modify their song elements in adulthood, any stress-induced deficits in song learning during early life may reliably indicate how well an individual was able to develop in the face of stress (e.g. Spencer et al. 2003).

One way in which external environments can affect development is through glucocorticoids via activation of the hypothalamic-pituitary-adrenal (HPA) axis. Glucocorticoid receptors (GR) are present in the song control nuclei HVC (proper name) and RA (robust nucleus of the arcopallium) of the avian brain, implying that these areas are potential targets for glucocorticoids such as corticosterone (CORT; Shahbazi et al. 2011; Suzuki et al. 2011). Artificially increasing CORT during development reduces song complexity, song learning, and HVC volume (Nowicki et al. 2002; Spencer et al. 2003; Buchanan et al. 2004; MacDonald et al. 2006; Shahbazi et al. 2011; Schmidt et al. 2013.). Furthermore, song sparrows (*Melospiza melodia)* with larger song repertoires had larger HVC volumes and lower CORT concentrations in response to stress (Pfaff et al. 2007; MacDougall-Shackleton et al. 2009). Collectively, these findings suggest that CORT may be a mechanism by which developmental stress affects song.

The timing of developmental stress may be particularly important because stressors will likely have greater or weaker effects on traits depending on the timing of their development relative to the timing of the stressor (Spencer & MacDougall-Shackleton 2011). For instance, altering nutritional conditions during the age when cheek patches of male zebra finches are developing affected cheek patch expression, but altering nutritional conditions before that age did not (Honarmand et al. 2010; Naguib & Nemitz 2007). Previous experimental studies

on developmental stress and song using zebra finches usually terminate treatment once birds reach nutritional independence, around post-hatch day (PHD) 30 (e.g. Spencer et al. 2003; Gil et al. 2006; Holveck et al. 2008). However, zebra finch song control regions HVC, RA and Area X develop and mature from approximately PHD 10 – 50 (Bottjer et al. 1985), coinciding with the behavioral development of song learning, which extends well past PHD 30. During a sensory phase (PHD 25-65) young birds memorize song(s) from adult tutors, and during a subsequent sensorimotor phase (PHD 30-90) young birds practice matching their vocal output to the memorized tutor songs (Konishi 1965; Marler 1970; Brainard & Doupe 2000; Immelmann 1969; Slater et al. 1988). Thus, developmental stress after PHD 30 should likely affect zebra finch song development.

In addition it is unclear whether different aspects of song can convey information about different periods of development. In zebra finches, stress during the sensory phase can affect aspects of song learning (such as imitation of tutor song and complexity of song), presumably by influencing neural representation of tutor songs (e.g. Spencer et al. 2003; Zann & Cash 2008; Holveck et al. 2008). On the other hand, stress during the sensorimotor phase may have more potent effects on vocal performance because during this phase birds must learn to precisely coordinate motor movements to match the neural representation of song (Podos et al. 2009; Sakata & Verhencamp 2012). Vocal performance includes aspects of song that are physically difficult to produce, such as consistent or stereotyped renditions of a song, and increased trill rates that place great demands on birds' ability to perform rapid modulations of the syrinx, respiratory, and vocal tract motor systems (Podos et al. 2009).

Preventing birds from singing during the late sensorimotor phase impaired adult song stereotypy (Pytte & Suthers 2000). These results imply that developmental stress may affect vocal performance if it reduces time spent practicing singing. As described above, previous work shows that zebra finch song learning may be affected by developmental conditions, but whether vocal performance is also affected remains unresolved. So far, song rate has been the principle measure of the effects of developmental stress on vocal performance in zebra finches (Birkhead 1999; Spencer et al. 2003; Gil et al. 2006; Brumm et al. 2009; Tschirren et al. 2009), but this measure may not be representative of true vocal performance capabilities (Podos et al. 2009). For instance, song rate may be affected by current state of motivation

(see Heimovics & Riters 2005). Thus, additional studies on whether developmental stress affects more comprehensive measures of vocal performance in zebra finches are required.

Changes in timing of nutritional stress open the possibility of catch-up growth. Catch-up growth refers to a period of accelerated growth following a period of nutritional deprivation and has been shown to affect various physiological and behavioral aspects of phenotype (Metcalfe & Monaghan 2001). The costs of accelerating growth may be immediate or delayed, and may in fact contribute unique effects above and beyond poor nutritional conditions alone. For example, rapid growth is associated with greater levels of oxidative stress and reduced investment in protein maintenance, which suggests that catch-up growth may shift investment into growth and reduce investment in somatic maintenance and repair (Metcalfe 2003). In birds, catch-up growth can affect learning and behavior (Fisher et al. 2006; Krause et al. 2009; Krause & Naguib 2011; see chapter 4), but whether song is affected directly by developmental stress or indirectly by catch-up growth requires further investigation.

Our aim was to determine the relationship between timing of the developmental stress and adult song complexity and song performance in zebra finches, and to determine whether CORT mediates the effects of stress on song. More specifically, we addressed the following questions: (1) Does stress at different developmental periods have distinct effects on HPA axis? (2) Does stress during later juvenile development (PHD 30-60, when young are feeding independently) affect adult song learning and/or vocal performance and if so, can it be distinguished from the effects of early stress? (3) Does catch-up growth affect song learning and vocal performance? To address these questions we manipulated diets of offspring zebra finches during early and/or juvenile development (i.e., pre- and/or post-nutritional independence). We tested HPA axis function during diet manipulations and afterwards in adulthood. Once male offspring reached adulthood, we also recorded song and quantified song complexity, learning accuracy, stereotypy and output. Our results indicated that nutritional manipulations may have long-term effects on HPA-axis function, but that these effects are not reflected in any of the song parameters quantified.

5.3 METHODS

5.3.1   Animals and manipulation

These birds were the same birds that we collected data from in chapter 4. To summarize the feeding treatment used, we manipulated food accessibility during two different phases: the early phase (PHD 5 – 35), and the juvenile phase (PHD 36 – 62). There were two feeding treatments: birds in the high feeding treatment (H) were given access to 65g seed and 13.5g egg-food daily, while broods in the low feeding treatment (L) were given access to 50g total of seed in a mixture containing a 1:3 ratio of seeds and woodchips (by volume), and 6.5g egg-food daily. This manipulation has been used by many others and has been shown to negatively affect body mass, adult song control brain regions, and song characteristics of zebra finches (Lemon 1993; Spencer et al. 2003; Buchanan et al. 2004; Zann & Cash 2008). We raised offspring zebra finches on H or L feeding treatment during the early phase and then switched feeding treatments for half of the birds in each of the H and L groups during the juvenile phase. This resulted in four feeding treatments: HH, HL, LH and LL. After the juvenile phase, all birds were given *ad libitum* seed. Offspring were kept with their parents until PHD 90 to ensure that young males learned song from their fathers exclusively (Adret 1993; song data were collected for chapter 5) and then housed in same-sex groups of four to five birds. All birds were at least 100 days of age before being subjected to any of the behavioral tests.

This study was conducted over two years (2011 and 2012). The start and end of feeding treatment differed very slightly between the two years: in the first year of the study (2011), experimental treatment began when the oldest nestling within a nest was PHD 6 and lasted until approximately PHD 61. In the second year of the study (2012) the treatment duration was PHD 5 through PHD 62). Potential year effects were controlled statistically (see below). A total of 9 and 13 breeding pairs produced 33 and 58 experimental offspring in the first and second year of the study, respectively. Sample sizes for each group after combining both years were: HH = 20 (10 males), HL = 27 (13 males), LH = 23 (9 males), LL = 21 (13 males).

5.3.2 Song recording and analysis

Songs were recorded from males after song had crystalized (Immelmann 1969) at approximately PHD 200 (mean and SD = 215.86 ± 9.97) and PHD 100 (mean and SD = 107.29 ± 12.39) in the first and second year of the study, respectively. Males were isolated in sound attenuation chambers for 24 h and then an unfamiliar female was placed in a cage next to the male and his directed song was recorded for 10 min using a Marantz PMD 670 recorder and an omnidirectional microphone (Sennheiser ME 62). After the recording session ended, birds were returned to their home cages and the same procedures were repeated again approximately one week later. Songs were digitized with a sample frequency of 44.1kHZ and a bit rate of 32 bits per second and sound spectrograms were generated using Raven Pro 1.4 software.

Zebra finch song is composed of a stereotyped sequence of syllables, usually preceded by repetitions of a simple introductory syllable. Syllables are separated by periods of silence of less than 5 ms (Sossinka and Böhner 1980), and syllables are themselves composed of elements that can be separated by silence or by abrupt changes in frequency modulation or amplitude (Williams and Staples 1992). Zebra finches may also sing many songs consecutively (song bouts) and these bouts of singing are defined as periods of singing with less than 2 s of silence between songs (Sossinka & Böhner 1980).

Song complexity: Zebra finches may sing variations of their song by adding, repeating, or skipping elements or syllables in their song (Sturdy et al. 1999), so we chose the most common variant amongst 10 randomly selected songs as the birds' primary song. Using the primary song we visually identified the number of elements and syllables as well as number of unique elements and syllables. We did not count the number of introductory elements towards the total number of elements in a bird's song.

Learning accuracy: We also calculated three measures of learning accuracy. The first measure was the similarity index (Böhner 1990, Eales 1985; Clayton 1987), which quantifies the number of elements the tutor and tutee song have in common. The value of the similarity index varies between 0 and 1, where 1 indicates that the number of elements in the two songs is identical. We calculated the similarity index once for each tutor – tutee pair by comparing the most common variant (i.e. primary song) of the tutor song to the most common variant of

the tutee song. The second measure of learning accuracy was degree of syllable match (i.e. the degree of similarity between a syllable copied by tutee and the original syllable produced by the tutor). After visually identifying which syllables a tutee had copied from their tutor, we isolated these syllables from 10 renditions of the tutee's primary song and cross-correlated them with the corresponding syllable in 10 renditions of the tutor's primary song. Syllables in the tutee song that were not copied from the tutor song were not included in the cross-correlation analysis. So if a tutor's primary song consisted of ABCD syllables and a tutee's primary song consisted of $A_1B_1C_1E_1$ syllables, then we quantified similarity of frequency spectrograms of A against A_1, B against B_1 and C against C_1. Cross-correlations were performed using Raven Pro 1.4 batch correlations function. The last measure of learning accuracy was precision of syntax learning used by Holveck et al. (2008). By examining transitions between elements, this measure quantifies how accurately tutees were able to arrange elements they had learned in the same order as the tutor's song. The total number of shared transitions between the tutor and tutee primary songs was divided by the total number of shared elements minus 1 to obtain a value between 0 and 1 that indicates the proportion of shared transitions (see Appendix B in Holveck et al. 2008 for complete algorithm). A value of 1 indicates that all the transitions between the same elements in the two songs were shared, thus indicating that the tutee was able to perfectly replicate the sequence of copied elements.

Song stereotypy: We calculated four measures of song stereotypy to assess vocal performance. The first two measures, sequence consistency and sequence linearity, examine the stereotypy of syllable sequencing. Sequence consistency expresses the frequency with which a common sequence appears and is calculated as the proportion of syllable transitions that conform with the most frequent transition for a given syllable (Scharff & Nottebohm 1991; Kao & Brainard 2005). For example, if a bird's primary song consists of syllables ABCD, but in one randomly selected song he sings BACD, then the sequence consistency score for this comparison would be 0.33 (only the transition from C to D match). For this study, we define sequence consistency as the proportion of syllable transitions in 10 randomly selected songs that conform to the syllable transitions of a bird's primary song. Sequence linearity quantifies the number of different possible transitions that can be observed after each unique syllable (Foster & Bottjer 2001; Kao & Brainard 2005). Both

sequence consistency and linearity yield a score between 0 and 1, with 1 indicating complete consistency or linearity. However, unlike sequence consistency, sequence linearity is not affected by variability at the end of songs. Therefore, our third measure of song stereotypy was the percentage of syllable types that terminated songs (Kao & Brainard 2005). Because these three measures are sensitive to the number of songs analyzed, we limited analyses to 10 randomly selected songs per bird. The fourth measure of stereotypy was syllable consistency, which we defined as the similarity between all renditions of a syllable produced. We cross-correlated all renditions of a syllable produced using the batch correlation function in Raven Pro 1.4.

Song output: We calculated three measures of song output: total number of songs, latency to sing, and maximum song bout duration. Total number of songs included the bird's primary song and any variants.

5.3.3 HPA axis function

Effects of nutritional stress on the HPA axis function were examined during early and juvenile phases, as well as in adulthood. Consequently we have corticosterone (CORT) samples for two time points during development (PHD 30 and 60) while birds were still on treatment, and one sample for a time point after treatment was terminated (PHD 240). For HPA axis function we collected data from both sexes.

Stress series: Elevation of CORT in response to an acute stressful situation was quantified in birds in both the first and second year of the study (2011 and 2012), using a standardized capture-restraint protocol (Wingfield et al. 1995). The samples were collected when the average age of all the offspring in the nest was PHD 30 and 60. Between 09:00 and 11:00, blood samples were collected from the brachial vein within 3 min of disturbance to obtain baseline CORT levels. Birds were then placed in cloth bags and blood was collected again after 15 and 30 min to characterize elevation and peak CORT levels (Wada et al. 2008). In total, approximately 75 μL of blood was collected from an individual during one series of blood sampling.

ACTH and DEX challenge: ACTH and DEX measures of HPA axis function were obtained only from birds in the first year of study (2011). At approximately PHD 240, we tested

whether nutritional stress during development had long-term effects on HPA-axis functioning by measuring maximum CORT output and the strength of the negative feedback using standardized doses of adrenocorticotropic hormone (ACTH) and dexamethasone (DEX), respectively (following Dickens et al. 2009). Doses of 25 IU/kg porcine ACTH dissolved in Ringers solution (Sigma Aldrich, A6303) and 1000 µg/kg DEX (Sandoz Canada Inc, 2301) were injected into the pectoralis muscle using a 300 µL insulin syringe. The appropriate injection volume to achieve the desired dose was calculated based on individual body mass measured on the previous day. Similar concentrations have been successfully used by Schmidt et al. (2012) to increase and suppress CORT in song sparrows (*Melospiza melodia*). Our own pilot studies in zebra finches indicate that there were minimal differences between using 25 IU/kg and 100 IU/kg ACTH, so we used the lower dose. For DEX, we determined that a dose of 1000 µg/kg was more effective at suppressing CORT than a dose of 500 µg/kg.

ACTH and DEX challenges were conducted on separate occasions, with two weeks between the challenges. For both ACTH and DEX challenges, a baseline blood sample was collected within 3 min of disturbance and a stress-induced sample was collected after 30 min of restraint stress prior to injections. Immediately after the second blood sample, birds were injected with ACTH or DEX and then released into individual cages with access to water. Post-injection samples were collected 30 min after injections (i.e. 60 min after initial disturbance). Therefore we had CORT samples from three different time points: within 3 min (baseline), 30 min (pre-injection), and 60 min after disturbance (post-injection) from both ACTH and DEX challenges. In total, approximately 100 µL of blood was collected from an individual during each of the challenges. Plasma was separated from all samples and stored at -20° C until assayed.

Corticosterone assay: Samples from both 2011 and 2012 were assayed simultaneously. CORT was quantified in unextracted plasma using a radioimmunoassay (MP Biomedicals, 07-12013) that has been previously validated in zebra finches (Schmidt & Soma 2008). Plasma was diluted 1:10 with steroid diluent (5 µL plasma + 45 µL diluent) and samples were run in duplicate (50 µL assay volume). Samples were analyzed randomly with respect to treatment in 8 assays. We included a sample from a pool of zebra finch plasma in every assay to measure inter-assay variability. The lowest point on the standard curve was 1.56 pg/tube and the highest point on the standard curve was 250 pg/tube. All samples fell within

the range of the standard curve (1.56-250 pg/tube). The intra-assay coefficient of variation was 11.53% for a low control (12.5 pg/tube) and 4.78% for a high control (125 pg/tube). The inter-assay coefficient of variation was 7.89% for the low control and 10.54% for the zebra finch plasma pool.

5.3.4   Statistics

Statistical analyses were conducted using SPSS 20.0. We used linear mixed models with restricted maximum likelihood (REML) to determine the effect of treatment on all our dependent measures (i.e., song and CORT). This analysis is appropriate for our data because we can control for the potential nonindependence of our samples (i.e. relatedness of siblings in each nest) to avoid pseudoreplication. In all of the analyses described below we first tested the significance of the random effects of broods and individuals nested in broods by using maximum likelihood theory to compare fitting of data into a similar model without the random effect. As these random effects did not contribute significantly to all models, some results are reported with these random effects excluded from the final model. All two-way and higher-order interactions were included in the full model and stepwise deletion of non-significant terms was applied to obtain the most parsimonious model of the data. Pairwise comparisons between treatment groups were adjusted using Sidak corrections.

Song learning and vocal performance: We used factor analysis to reduce the number of song variables because many of the variables within each parameter (i.e. complexity, learning accuracy, stereotypy, and output) were correlated. Table 5.1 shows the results of the principal components analyses (PCA) and their factor loadings for each song parameter. For song complexity, we first obtained residuals from the regression of tutee elements and syllables against tutor elements and syllables (and similarly for unique elements and syllables). PCA of these four residuals extracted one component that explained 75.61% of the total variance. All song measures had high positive loadings on this component. PCA of measures of song learning accuracy also extracted one component that explained 64.01% of the total variance. Proportion of shared transitions and similarity index showed high positive loadings while degree of syllable match showed modest positive loading on this factor. PCA of song stereotypy and output measures also resulted in one component each that explained 55.21% and 65.22% of the total variance, respectively. For both components, measures showed high

Table 5.1. Principal components analysis (PCA) for song complexity, learning accuracy, stereotypy, and output

	% total variance	factor loadings on extracted component
song complexity	75.61	# elements (0.928)
		# syllables (0.915)
		# unique elements (0.892)
		# unique syllables (0.728)
song learning accuracy	64.01	shared transitions (0.968)
		similarity index (0.951)
		syllable match (0.280)
song stereotypy	55.21	sequence consistency (0.879)
		sequence linearity (0.762)
		% terminating syllables (-0.540)
		syllable consistency (0.751)
song output	65.22	total # songs (0.825)
		latency to sing (-0.759)
		max. song bout duration (0.836)

Note: Numbers in brackets indicate factor loadings for extracted component. Separate principal components analyses were conducted for each measure of song learning and vocal performance (song complexity, learning accuracy, stereotypy, and output).

positive loading except for % syllables terminating song and latency to sing, which had negative loadings on stereotypy and output, respectively.

To analyze effects of our manipulation on song learning and vocal performance, separate linear mixed models were used with each component obtained from the PCAs as the dependent variable. Feeding treatment was entered as a fixed effect, with age at time of song recording as a fixed effect covariate. Year of the study (2011 or 2012) was entered as an additional random effect. The total sample size was 44 because we were unable to obtain any songs from one male in the HH group despite multiple recording attempts.

HPA axis function: We calculated three different measures of HPA axis function: baseline CORT (early phase, juvenile phase, and ACTH and DEX challenges before injections), integrated CORT response of samples collected during the early and juvenile phases, and HPA challenge. Both males and females were included in analyses of baseline CORT, integrated CORT, and HPA challenge. Integrated CORT response was defined as the area under the curve created by plotting values of the baseline, 15 min, and 30 min samples and provides information on CORT increase and clearance rates (Breuner et al. 1999). Area under the curve was calculated using Prism 5.0 (Graphpad software). HPA challenge was defined as change in CORT for ACTH and DEX challenges, and was calculated as the difference between the 60 min and 30 min sample (we refer to this difference as ACTH change and DEX change).

Separate linear mixed models were used for baseline CORT, integrated CORT, ACTH change, and DEX change. The binary variable, year of study (2011 or 2012), was entered as an additional random effect for all models. For baseline CORT, baseline values from all sampling sessions (i.e. early phase, juvenile phase, ACTH challenge, and DEX challenge) were entered as the dependent variable, with feeding treatment, sex, and sampling session as fixed effects. For integrated CORT separate linear mixed models were used for integrated CORT responses at the pre- and post-nutritional independence phases. For both models, integrated CORT (ng/mL per min) was entered as the dependent variable, with group and sex as fixed effects. For HPA challenge, ACTH change and DEX change were entered as the dependent variable, with feeding treatment and sex as fixed effects. Sample sizes for ACTH change and DEX change were HH=6, HL=10, LH=9, LL=8.

Table 5.2 summarizes the dependent variables, fixed, and random effects used for each of our measures of HPA function and song learning and performance.

5.4 RESULTS

5.4.1 Song learning and vocal performance

Nutritional stress during development did not significantly affect any measure of song learning or vocal performance (figure 5.1). There was no significant main effect of feeding treatment on principal component factors of song complexity ($F(3, 40) = 0.80$, $p = 0.502$), learning accuracy ($F(3, 40) = 0.36$, $p = 0.780$), song stereotypy ($F(3, 40) = 0.084$, $p = 0.968$), or song output ($F(3, 13.62) = 1.87$, $p = 0.183$).

5.4.2 HPA axis function

Baseline CORT: Diet manipulations had significant effects on baseline CORT only during development. Analyses indicated that there was no significant main effect of feeding treatment (overall means & SEM for HH = 5.86 ± 1.32; HL = 6.35 ± 1.34; LH = 4.98 ± 1.38; LL = 7.13 ± 1.39), however, there was a significant main effect of sampling session ($F(3, 179.35) = 14.99$, $p<0.001$) and a significant interaction of feeding treatment × sampling session ($F(9, 186.76) = 5.61$, $p<0.001$). Pairwise comparisons of the interaction indicated that during the early phase, HL had significantly lower baseline CORT than LL ($p=0.037$), and almost significantly lower baseline CORT than LH ($p = 0.052$; fig. 2). During the juvenile phase, HH and LH had significantly lower baseline CORT than HL and LL ($p<0.01$ for all; figure 5.2). Baseline CORT levels in adulthood (ACTH and DEX baseline samples) were not affected by feeding treatment. This suggests that L treatment temporarily elevated baseline CORT during development, but these effects did not persist after diet manipulations ended.

Integrated CORT response: Feeding treatment significantly affected integrated CORT responses at the post-nutritional independence phase only (Fig. 3). Analyses of integrated CORT responses at the pre-nutritional independence phase revealed that there were no significant main effects of feeding treatment or sex ($F(3, 16.93) = 1.53$, $p = 0.243$ and $F(1, 79.63) = 3.82$, $p = 0.054$, respectively), or significant interaction of feeding treatment × sex ($F(3, 78.31) = 2.12$, $p = 0.104$). However, analyses of the integrated CORT responses at the post-nutritional independence phase indicated that there was a significant main effect

Table 5.2 Summary of fixed and random effects of the linear mixed models for analysis of song and HPA function

Fixed covariates are italicized. Random effects that were retained in the final model are marked with an asterisk (*). The random effect individuals nested in broods is abbreviated as indiv(brood). Study year was entered as the binary value 2011 or 2012.

	Dependent variable	**Fixed effects**	**Random effects**
Song learning & vocal performance (song complexity, learning accuracy, stereotypy, output)	PCA factor scores	feeding treatment *age[1]*	brood[2]* indiv(brood) study year
Baseline CORT	baseline CORT concentrations (ng/mL)	feeding treatment sex sampling session[3]	brood* indiv(brood)* study year[3]
Integrated CORT response	integrated CORT response (ng/mL per min)	feeding treatment sex sampling session[4]	brood* indiv(brood) study year[3]
HPA reactivity	ACTH and DEX change (ng/mL)[5]	feeding treatment sex	brood indiv(brood)

[1]Age at time of recording. [2]Significant for song output only. [3]Refers to baseline samples collected from the early phase, juvenile phase, ACTH challenge, and DEX challenge. [4]Refers to integrated CORT responses from the early and juvenile phase. [5]We used separate linear mixed models for ACTH change and DEX change.
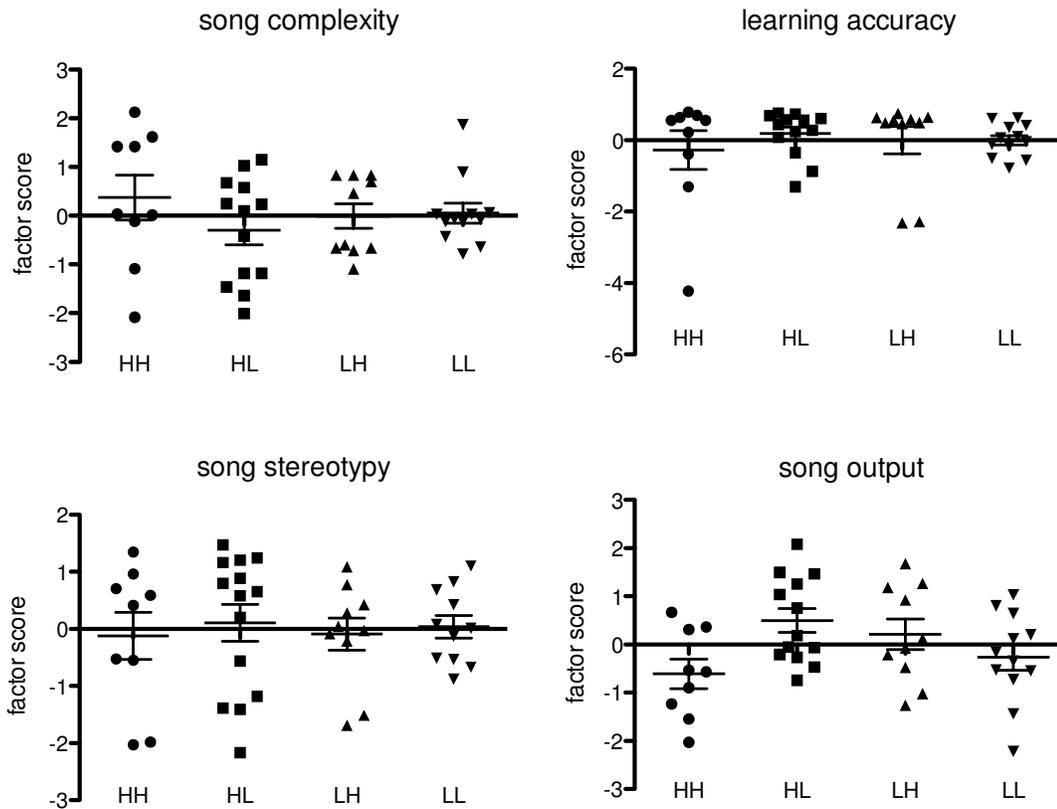
Figure 5.1 Main effects of feeding treatment on song learning and performance in adult males

Feeding treatment did not significantly affect measures of song learning and vocal performance. Horizontal lines indicate mean, error bars represent ± 1 SEM. Points indicate raw individual data (uncorrected for brood size or year of study effects).

Figure 5.2 Interaction of feeding treatment × sampling session on baseline CORT during development and in adulthood

During the early phase HL had significantly less CORT than LL (indicated by *). During the juvenile phase HH had significantly less CORT than LL and HL (indicated by #s). Groups did not significantly differ in baseline CORT when sampled as adults (ACTH and DEX sessions). Error bars are ± 1 SEM.

of feeding treatment (F(3, 78) = 4.32, p = 0.007), but no significant main effect of sex or interaction of feeding treatment × sex. Pairwise comparisons of the significant main effect indicated that the HL group had significantly higher integrated CORT responses than the HH group during the post-nutritional independent phase ($p = 0.005$; figure 5.3). This result suggests that L conditions during the post-nutritional independence phase lead to higher rates of CORT secretion in birds that had previously (i.e. in the pre-nutritional independence phase) experienced H conditions.

HPA challenge: Feeding treatment significantly affected CORT concentrations in the ACTH, but not DEX challenge. Analysis of ACTH change revealed a significant main effect of feeding treatment ($F(3, 22) = 4.64$, $p = 0.012$; figure 5.4). Pairwise comparisons indicated that the LL group had significantly lower change in CORT following ACTH injection than HH and LH ($p = 0.025$ and 0.033, respectively). This indicates that LL conditions reduced ACTH-induced adrenal cortex sensitivity to ACTH and/or capacity to secrete CORT. DEX change was not significantly affected by diet manipulations (group $F(3, 25) = 0.56$, $p = 0.648$; figure 5.4).

5.5 DISCUSSION

The current experiment investigated the effects of nutritional stress at different developmental periods on HPA axis function, song learning and vocal performance. Our results indicate that nutritional stress did not affect song learning or vocal performance, despite temporarily elevating baseline CORT concentrations. Similarly, increased rate of CORT secretion during development did not seem to have long-term effects on HPA axis function or song. However, LL conditions appeared to decrease sensitivity of adrenals to ACTH and/or ability to secret CORT. There was no difference between early and juvenile nutritional stress on song learning or vocal performance. Our results also suggest that neither HPA axis function nor song measures were affected by catch-up growth.

To the best of our knowledge, this study provides the most comprehensive assessment of the effects of developmental stress on zebra finch song. Surprisingly, nutritional stress did not significantly affect any measures of song learning or vocal performance used in the current experiment, which is inconsistent with the developmental stress hypothesis that song is an honest indicator of developmental history (Nowicki et al. 1998, 2002; Nowicki & Searcy
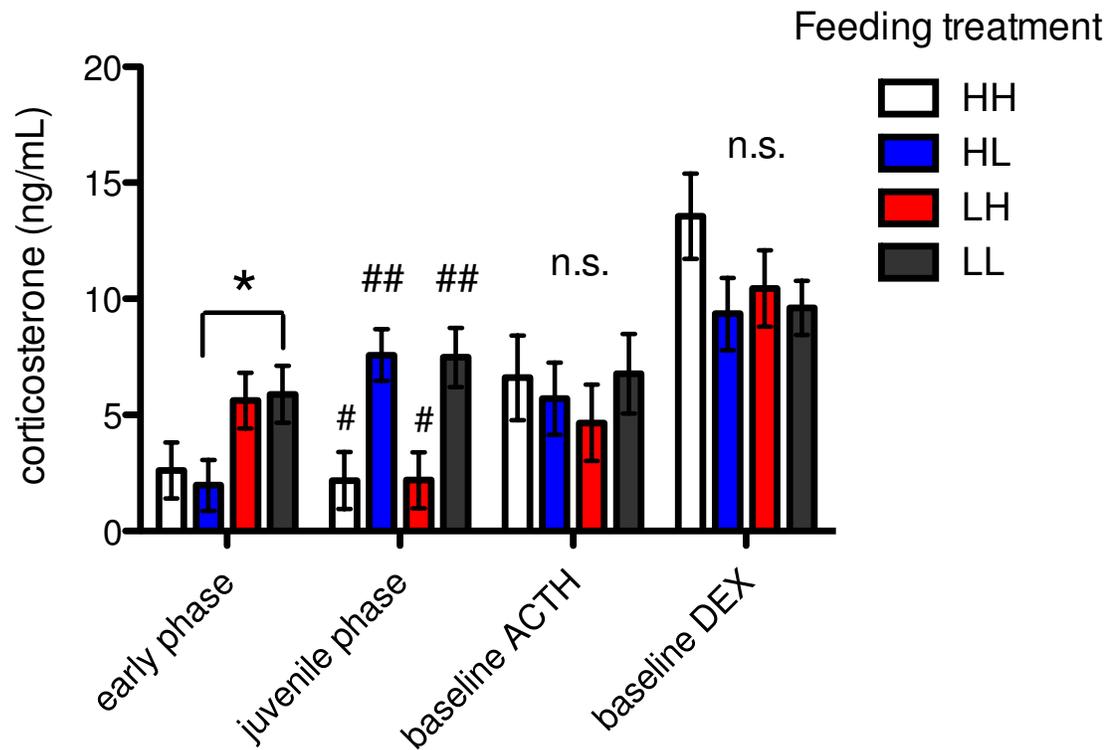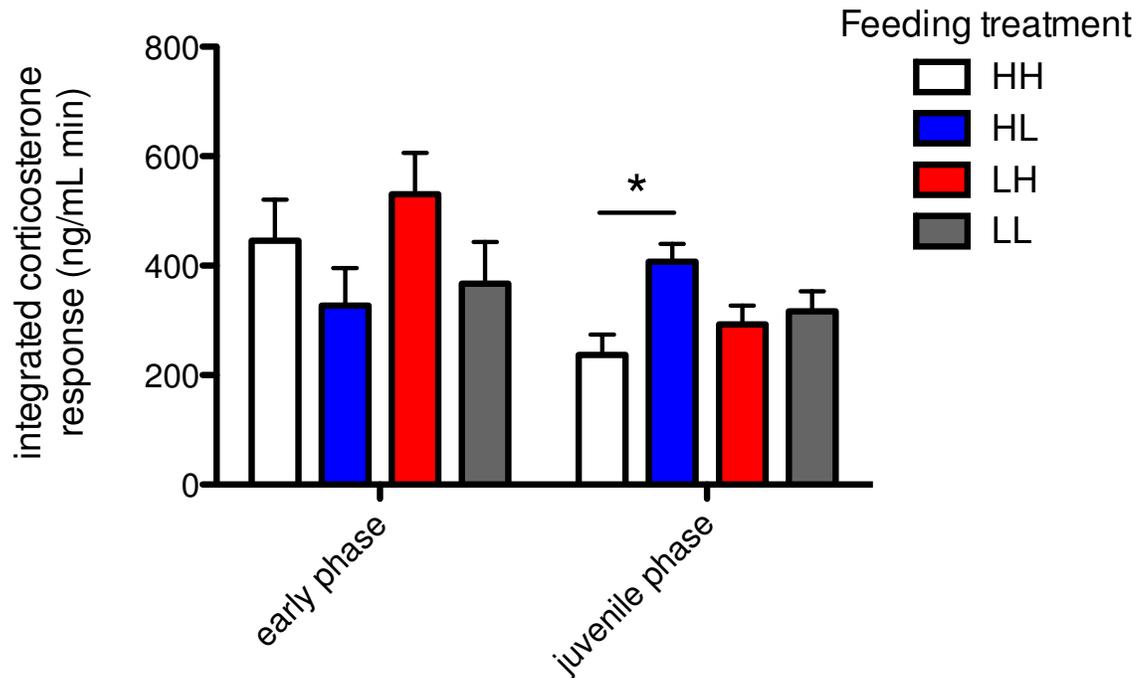
Figure 5.3 Main of feeding treatment on integrated CORT response in the early and juvenile phases

Feeding treatment did not significantly affect integrated CORT response during the early phase. However, feeding treatment significantly increased integrated CORT response of the HL group compared to HH group during the juvenile phase. Error bars are ± 1 SEM.
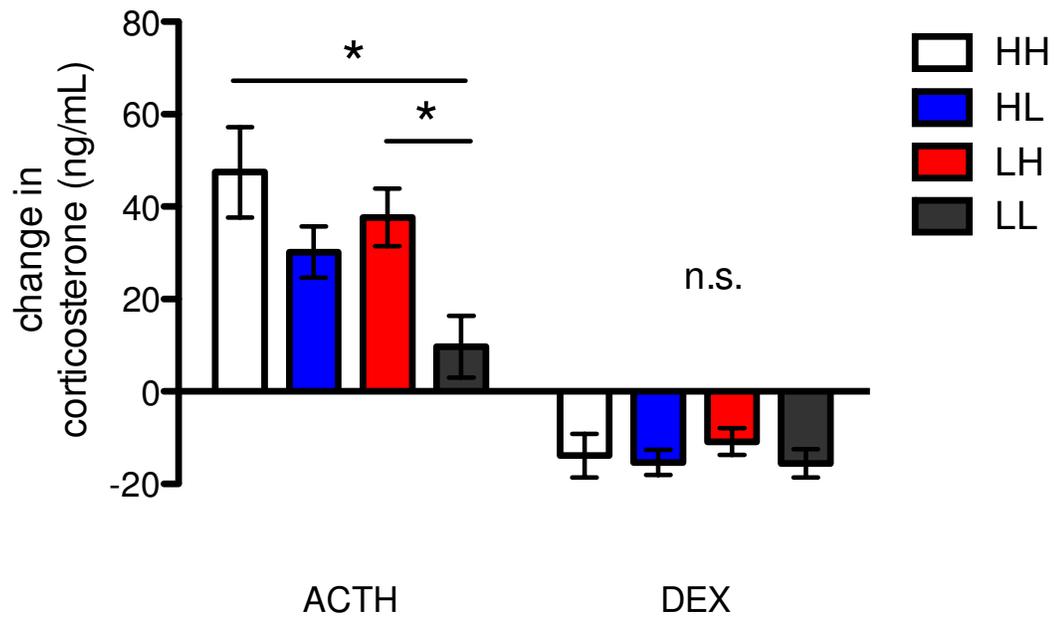
Figure 5.4 Main effect of feeding treatment on change in CORT for ACTH and DEX challenges.

The LL group secreted significantly less CORT compared to HH and LH groups in response to ACTH injections. However, there were no differences between groups in response to DEX injections. Error bars are ± 1 SEM.

2004). In zebra finches, experimental studies on whether developmental stress affects song have yielded inconsistent results. Similar food restriction protocol as this study and artificially elevation of CORT in nestlings until PHD 30 both reduced song complexity and HVC volume (Spencer et al. 2003; Buchanan et al. 2004). The same food restriction manipulation was used by Zann and Cash (2008) and Brumm et al. (2009). Like Spencer et al. (2003), Zann & Cash (2008) also found that stress reduced song complexity. In addition, they reported that stress had no effect on a measure of learning accuracy. On the contrary, Brumm et al. (2009) reported that stress had no effect on song complexity or song amplitude, but reduced the accuracy of syllable sequence matching. Reduced syntax matching but no effect on song complexity was also reported by Holveck et al. (2008), who used differences in brood size as the developmental stress manipulation. Gil et al. (2006) also manipulated brood size, but found that it had no effect on song complexity, learning accuracy, or volumes of song control nuclei HVC, RA and LMAN (lateral part of nucleus mangnocellularis anterioris).

Effects of developmental stress on song rate are just as perplexing, as song rate was not affected by diet quality in two experiments (Birkhead et al. 1999; Spencer et al. 2003), but was increased by both enlargement and reduction of brood sizes in two separate experiments (de Kogel & Prijs 1996; Tschirren et al. 2009). Even current nutritional conditions have been reported to affect song rate (Ritschard & Brumm 2012). Our findings make a novel contribution in addition to defending Gil et al.'s (2006) report of a lack of effect of early developmental stress on song by indicating that zebra finch song learning and vocal performance (using more comprehensive measures that includes song rate) are not affected by early or juvenile nutritional stress. We acknowledge that the age that we chose to manipulate juvenile stress corresponded to an age where there was an overlap between the sensory and sensorimotor phase, thus effects of juvenile stress on song performance might have been clearer if stress was experienced later in development (i.e. PHD 80-90), such as the one used by Pytte & Suthers (2000). In light of the inconsistencies in the literature mentioned above, more studies are almost certainly needed to confirm our findings.

The inconsistent effects of developmental stress on zebra finch song do not discredit the hypothesis that song may be an accurate indicator of developmental aspects of male quality (Nowiciki et al 1998, 2002; Nowicki & Searcy 2004). Instead, it suggests that the reliability

of song as an indicator may be species-specific due to the way song is used. Zebra finch courtship involves a combination of song and displays from the male. Song and courtship displays are produced in close proximity to females, which allow her to visually assess other traits such as beak color and chest symmetry in addition to song characteristics (Collins et al. 1994; Swaddle & Cuthill 1994; Zann 1996). As females likely assess many traits in order to gain information about multiple aspects of male quality (Keagy et al. 2012), male song may be a supplementary, less important factor in mate assessment. In fact, females seem to show stronger preference for redder beak color than high song rate, although these two traits are not always correlated (Collins et al. 1994; Birkhead et al. 1998; Simons & Verhulst 2011). In the absence of visual cues, however, female zebra finches may turn to using song rate, structure, and complexity, which may explain why they can show preference for these aspects of song (Riebel 2009).

On the other hand, song repertoire size of song sparrows appears to be a much more reliable indicator of male quality (correlating positively with body condition, survival and reproduction, and cognitive abilities) and is reduced in birds that experience developmental stress (e.g. Pfaff et al. 2007; MacDonald et al. 2006; Schmidt et al. 2013; MacDougall-Shackleton et al. 2009; Sewall et al. 2013). This could be because song sparrows songs are broadcasted over long distances by territory-holding males in order to attract females. Consequently, male song should contain enough information to offset the costs for a female journeying to investigate a male in his territory.

Elevated baseline CORT in response to nutritional stress during development has been reported by some, but not all, other studies (e.g. Spencer et al. 2003; Constantini 2010; Honarmand et al. 2010). In the present study we found that L conditions elevated baseline CORT, but that these effects were temporary and did not persist into adulthood. However, even though baseline CORT was not elevated in adulthood, persistent developmental stress did affect adult response to HPA challenges. Increased integrated CORT response of HL birds during the juvenile phase also did not have effects on responses to HPA challenges in adulthood. Birds in the LL group secreted significantly less CORT in response to adrenal stimulation by ACTH. Chronic stress can decrease HPA axis reactivity to ACTH by down-regulating adrenal ACTH receptors, or diminishing capacity of adrenals to produce CORT (Rich & Romero 2005). These adjustments likely help to mitigate the detrimental effects of

constantly elevated plasma CORT. Therefore, our results suggest that prolonged nutritional stress during development can have programming effects on HPA axis function.

Our results indicate that any catch-up growth exhibited by the LH group was not associated with short-term increases in CORT or long-term changes in HPA axis function. However, females in the LH group performed worse on an associative learning task (see chapter 4), suggesting that the effects of catch-up growth on learning and behavior may be selective (not all aspects of learning are affected), sex specific (females more strongly affected than males), and not mediated by elevated baseline CORT or changes in HPA axis function. Although it is possible that we did not find a positive association between CORT and catch-up growth because CORT samples were collected after the rapid growth phase (see Kriengwatana et al. submitted), other studies have also not detected this relationship (Honarmand et al. 2010; Krause et al. 2009).

Other mechanisms such as oxidative stress may account for the effects of catch-up growth in zebra finches (Barnes & Ozanne 2011; Monaghan et al. 2009). Reactive oxygen species are generated as a by-product of mitochondrial energy production. Oxidative stress occurs when there are insufficient antioxidants to neutralize the reactive oxygen species, resulting in cellular damage. Rapid growth during the catch-up phase and other forms of early life stress (without explicit catch-up growth) may render organisms more susceptible to oxidative stress later in life (Blount et al. 2003; Alonso-Álvarez et al. 2007; Noguera et al. 2011; Bourgeon et al. 2011). Higher oxidative stress may carry costs of reduced investment in reproduction and immune function, and faster senescence (Monaghan et al. 2009). Increased oxidative stress has also been linked to cognitive impairment and neurodegenerative disorders, presumably because the brain is particularly vulnerable to oxidative stress (Liu & Mori 1999; Fukui et al. 2001; Leutner et al. 2001; Farooqui 2008).

In conclusion, developmental stress may have highly variable effects on zebra finch song are depending on the type of stressor, timing of stress, and the parameters used to define song learning and/or vocal performance. This suggests that nutritional stressors that have short-term effects on HPA function do not affect song development in zebra finches. As adult HPA function was not affected by nutritional stress during development, song quality may not accurately reflect HPA development in this species. The lack of relationship between catch-

up growth and CORT also makes it unlikely that circulating CORT is the mechanism by which catch-up growth affects physiology or cognition.

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CHAPTER 6

GENERAL DISCUSSION

The studies conducted for this dissertation addressed the effect of timing of developmental stress and the extent to which phenotypic plasticity is driven by a change in developmental conditions or by consistently poor developmental conditions without change. These experiments provide important contributions to the understanding of developmental plasticity by indicating that some aspects of phenotype can be significantly altered by nutritionally stressful conditions during early and/or juvenile development (i.e. pre- and post-nutritional independence), whereas other aspects may not be as plastic, or may be affected in a way that is highly variable across individuals.

In chapter 2 I tested whether the timing of nutritional stress influenced physiological measures in zebra finches. Results indicated that stress during the juvenile phase elevated adult body mass and innate constitutive immune function. Growth was affected only by juvenile nutritional conditions, and was positively correlated with adult immune response. In particular, H early conditions followed by L juvenile conditions (HL) resulted in elevated adult body fat and immune response against *E.coli* microbes.

In chapter 3 I tested whether the timing of nutritional stress affected behavioral and cognitive measures in adult zebra finches. Results indicated that prolonged stress throughout early and juvenile development (LL) impaired hippocampus-dependent spatial memory, while HL conditions hindered learning a spatial associative task. Females in the HL group also committed more perseverative errors, which indicates reduced behavioral flexibility. However, nutritional treatments did not affect neophobia.

In chapter 4 I tested whether catch-up growth resulting from L early conditions followed by H juvenile conditions (LH) affected adult body condition and learning. In this chapter I also attempted to clarify whether the associative learning results obtained in chapter 3 were due to deficits in spatial learning or to inability to inhibit a previously learned response. Results indicated that females in the LH group were slower to learn associative learning tasks, regardless of the type of task (i.e. color or spatial). Also contrary to results from chapter 3, perseverative errors were not affected by nutritional treatments. Nutritional treatments also

did not have long-term effects on body fat, only short-term increases in the HL group that experienced juvenile stress.

In chapter 5 I tested whether catch-up growth contributed unique effects to male song and HPA axis function compared to early nutritional stress. Results indicated that nutritional stress did not significantly affect song learning or vocal performance. L conditions elevated baseline CORT concentrations during development, but this effect did not persist into adulthood. The HL group also showed elevated rates of CORT secretion during restraint stress during the juvenile phase. However, adult DEX-induced negative feedback was not affected by nutritional stress. Nutritional stress only had significant effects on adult HPA function if it was prolonged, as the LL group showed dampened CORT levels in response to ACTH-induced adrenal stimulation.

Overall, these results emphasize the role of juvenile environments in permanently altering phenotype, individual differences in developmental plasticity, and the different life history strategies of males and females.

6.1 PHENOTYPIC PROGRAMMING DURING JUVENILE DEVELOPMENT

Much research on phenotypic programming has focused on mammals and the prenatal and perinatal period, but accumulating evidence indicates that adolescence is also a period of heightened developmental plasticity and sensitivity to environmental cues that can produce lasting consequences on phenotype (Andersen 2003; Andersen & Teicher 2008; Lupien et al. 2009). Results from Chapter 2 and 3 add to this growing literature by showing that zebra finches are able to make long-lasting changes to phenotype in response to juvenile conditions. Specifically, nutritional stress during the juvenile phase temporarily increased fat mass during development and increased adult body mass and fat mass. Poor juvenile conditions following good early conditions (HL) also improved innate constitutive immune function but hindered associative learning and spatial memory. This suggests that a decline in juvenile environments may cause birds to tradeoff cognitive development for enhancement of physiological traits that likely increase chances of survival and reproduction.

Environmental mismatch hypotheses predict that the cause of a poorly adapted phenotype is a mismatch between past and present environments, as opposed to unfavorable past

environments *per se* (Monaghan 2008). Catch-up growth is one of the possible results of environmental change during development, which may benefit organisms in the short-run but impose costs that may manifest later as increased adiposity, insulin insensitivity, or reduced lifespan, amongst others (Metcalfe & Monaghan 2001; Hales & Ozanne 2003). Data from chapters 3 and 4 support the predictions of environmental mismatch hypotheses because associative learning was impaired by HL and LH conditions, respectively. Because switching from H to L conditions does not induce catch-up growth but still affected learning, this suggests that experiencing a change in developmental conditions may have more robust effects on cognition than consistently poor environments. However, interpreting these results as evidence of a maladapted phenotype is naïve because the tradeoff for reduction in cognition may have been extremely adaptive for short-term survival (i.e., increase in body size and fat storage).

Nevertheless, some traits may be more affected by prolonged stress than environmental mismatch. Data from chapter 3 and 5 indicate that HPA reactivity and hippocampus function were more strongly affected by consistently poor developmental conditions (i.e. cumulative stress) than by a change in conditions. Chronic stress may cause down-regulation of adrenal ACTH receptors or may reduce capacity of adrenals to produce CORT in order to ameliorate the potentially damaging effects of constantly elevated CORT (Rich & Romero 2005). The hippocampus may be particularly sensitive to stress-induced CORT elevations because glucocorticoids released in response to stress bind to receptors on the hippocampus to inhibit HPA axis activity (van Haarst et al. 1997). The impacts of stress during development on hippocampus development can be severe, as stress-induced reductions in hippocampus neurogenesis persist into adulthood (Lemaire et al. 2000; McEwen 2002a; Andersen & Teicher 2008; Tottenham & Sheriden 2009). The hippocampus is involved in learning and memory, and in birds, hippocampus development appears to be protracted (Clayton 1996). Consequently, longer-lasting elevation of circulating glucocorticoids during this developmental window may have greater effects on hippocampus. Overall, these results also emphasize that timing strongly influences which traits will be affected by developmental stress.

Perhaps one reason why chronic nutritional stress and the mismatch of early and juvenile nutritional stress produce different phenotypes is because they induce dissimilar epigenetic

modifications. Epigenetic influences of nutrition during development on phenotype are achieved by altering expression of specific target genes (Szyf 2009), therefore the differences in timing of stress may amplify or silence expression of a combination of a particular set of genes, or may affect expression of a different set of genes. Although it is uncertain which of these speculations applies to our findings, the emphasis here is that timing of stress may be able to induce diverse epigenetic changes that lead to different phenotypes.

If nutritional conditions in the juvenile phase are able to alter previous epigenetic changes acquired during the early phase, then DNA methylation or chromatin remodeling may still occur in later stages of development. Unfortunately, the relationship between adolescent stress and epigenetic changes has yet to be thoroughly investigated (see Hunter & McEwen 2013 for review of epigenetic changes across the lifespan). However, there is evidence that patterns of DNA methylation are dynamic and reversible. In hippocampal neurons, methylation and demethylation of may be part of the learning and memory processes (see Hunter & McEwen 2013). In fact, McGowen et al. (2008) suggest that nutrition in adulthood may even alter DNA methylation patters acquired during development. In the present series of experiments, all birds were maintained on the same diet after feeding treatment ended, which suggests that exposure to favorable conditions at a later age may not be sufficient to reverse effects of earlier unfavorable conditions. Evidently, reversing epigenetic changes require a more specific intervention regime, such as increases in certain micronutrients. For example, increases in brain methionine, an amino acid that can be obtained from dietary sources, can reverse stable maternal programming effects on glucocorticoid receptor expression and HPA axis responsiveness to stress (Weaver et al. 2009). Whether dietary methionine supplementation also has the same effect, and whether epigenetic alterations during juvenile or adolescent periods may also be reversed by this treatment is an exciting avenue for future research.

6.2 INDIVIDUAL DIFFERENCES IN DEVELOPMENTAL PLASTICITY

Data from chapter 3 and 4 indicate that a change in developmental conditions may affect associative learning and behavioral flexibility, however, the results are inconsistent between the two studies even though they used the same developmental manipulation and testing paradigm. Large individual differences in plasticity may explain this inconsistency. In the present studies, unmeasured aspects of development such as sibling interactions and parental

investment or favoritism, or *ad libitum* food supply in laboratory conditions after cessation of treatment may have contributed to individual differences in plasticity. Frankenhuis & Panchanathan (2011) explain that individual differences in plasticity would exist if: (1) it is adaptive for parents to produce offspring with varying levels of plasticity when the future is unpredictable and (2) frequency-dependent selection (conditions when the fitness of a phenotype is dependent on its frequency relative to other phenotypes in a population) maintains genetic variation in plasticity.

Individuals may vary in degree of phenotypic plasticity due to a combination of genetic factors and developmental experiences (Boyce & Ellis 2005). Specific genes or gene variants may predispose individuals to be more sensitive to environmental signals, and specific environmental contexts can have consistent effects on phenotype across individuals (Belsky et al. 2009; Belsky & Beaver 2011). For instance, parental insensitivity was found to correlate with externalizing behaviors in preschoolers (e.g. aggression, attention deficit hyperactivity disorder) only if the preschoolers possessed a dopamine receptor D4 polymorphism (Bakermans-Kranenburg & van IJzendoorn 2006). Moreover, this genetic difference also mediated the differential response of these children to behavioral intervention (Bakermans-Kranenburg et al. 2008). These and other studies demonstrate that more plastic individuals were at a disadvantage in unsupportive environments but were a greater advantage in nurturing environments (Belksy 2009; Belsky & Beaver 2011). This provides further support for the concept of behavioral syndromes, where early life experiences interact with genetic inheritance to affect the way individuals approach and deal with various environmental and situational challenges.

Individual differences in plasticity may also explain why some traits measured in the present studies (i.e., metabolic rates, neophobia, and song) appeared to be unaffected by developmental manipulations. Furthermore, development of one trait may impact development of other traits, which may lead to developmentally correlated traits. For instance, work in great tits (*Parus major*) has not shown that developmental stress affects exploratory or neophobia in adulthood, even though this relationship is evident in mammals (Naguib et al. 2011; Titulaer et al. 2012; Weinstock 2007). Development of these traits may be highly constrained due to canalization or developmental stability – processes that limit the effect of genetic or environmental variation on phenotype (e.g. Clarke 1998). However, the

latter explanation does not seem likely given previous research that metabolic rates, neophobia, and song can be altered by developmental stress (Verhulst et al. 2006; Krause et al. 2009; Krause & Naguib 2011; Banerjee et al. 2012; Spencer et al. 2003).

Frankenhuis & Panchanathan (2011) propose that individual differences in developmental plasticity may emerge if developmental trajectories are guided by stochastic sampling of environmental cues and the level of confidence that an individual has about the accuracy of those cues. This theory assumes that a certain threshold of confidence about the environment is required before phenotypic alterations are made, and that some cues may be inaccurate (e.g. whether environment is safe or dangerous). Due to stochastic sampling of cues, by chance some individuals will receive more congruent cues and reach confidence threshold earlier than others. Other individuals may take longer to alter phenotype because they receive heterogeneous, incongruent cues. Various correlations between traits subsequently occur because earlier or later modifications of a trait at a specific developmental stage can affect development of other traits (e.g. tradeoffs).

 In short, individuals with the same genotype living in the same environment may actually achieve different phenotypes because each individual receives a slightly different set of environmental cues that vary in accuracy. Individuals that are able to start tailoring their phenotype earlier may have greater fitness because they can become more specialized or adapted for the environment, but at the same time risk greater reduction in fitness if the environmental cues they received during development were inaccurate. Timing of exposure to particular cues presumably alters when the confidence thresholds are reached, thus affecting individual developmental trajectories and relationships between different traits.

6.3 SEX-SPECIFIC EFFECTS OF DEVELOPMENTAL STRESS

Across the present series of studies, HL and LH females showed greater sensitivity to effects of developmental stress on associative learning and behavioral flexibility, while HL males showed greater sensitivity to effects on hippocampus function (however, LL conditions impacted males and females similarly). These results suggest that timing of developmental stress may also have sex-specific effects on learning, which is a novel finding in relation to previous work showing that developmental stress can affect spatial learning and associative memory in birds (Fisher et al. 2006; Pravosudov et al. 2005; Farrell et al. 2012). These

findings also agree with previous work showing that female zebra finches are more susceptible to nutritional stress (de Kogel 1997; Martins 2004), and partially corroborate with evidence in mammals that developmental (prenatal) stress tends to alter prefrontal cortex and hippocampus structure and function in males, and anxiety, depression, and stress reactivity in females (Weinstock 2007). However, not all the studies examined by Weinstock reported sex differences in stress reactivity (e.g. Takahashi et al. 1998), which implies that individual differences in sensitivity to environmental cues may again mediate inconsistencies in stress reactivity. Furthermore, neophobia may not be an accurate measure of anxiety or depression, as it is commonly used as a measure of personality.

Sex differences in sensitivity to environmental conditions may arise from differential energy requirements in species that are sexually dimorphic in body size, from the presence of sex-linked recessive genes in the heterogametic sex, and from the actions of high levels of androgens required for male sexual development (Lindstöm 1999). Hormones may be especially important in mediating the effects of developmental stress on cognition. In particular, developmental stress may have sex-specific effects on associative learning by altering dopamine systems and their interactions with estrogens.

Early life stress can program dopaminergic circuits by affecting cell death, dopamine production and turnover, neuron morphology, and/or receptor expression and transmission (Rodrigues et al. 2011; Gatzke-Kopp 2011). Dopamine is important for associative learning tasks where animals must coordinate motor responses, create long-term memories, and keep track of consequences in order to modify future behaviors. This is because, among other things, dopamine neurons encode stimulus-reward associations and dopamine release acts as a 'prediction error' signal that compares expected outcomes with actual outcomes (reviewed in Asaad et al. 1998; Day et al. 2007). Premotor cortex, hippocampus, and prefrontal cortex are three key regions in regulating these processes (Asaad et al. 1998). The influence of dopamine on learning follows an inverted U-shaped curve, where hyper- or hypoactivity of dopaminergic neurons diminishes learning (Cools & D'Esposito 2011).

In the nidopallium caudolaterale (NCL, the avian analog of the mammalian prefrontal cortex; Güntürkün 2005; Herold et al. 2011), modification of dopamine levels can affect learning (e.g. Güntürkün & Durstewitz 2000). Pharmacological manipulations of dopamine can also

enhance or impair hippocampus-dependent functions, and conversely, hippocampus activity can influence dopamine release in different parts of the brain (Floresco et al. 2001; Jay 2003). At the same time, estrogens can also regulate learning processes related to prefrontal cortex and hippocampus (McEwen 2002b; Keenan et al. 2001). Moreover, in the hippocampus, estrogens have also been shown to be neuroprotective (Baudry et al. 2012). Therefore, developmental stress may affect dopamine-mediated learning, and the female-specific reduction in associative learning and male-specific reduction in hippocampus-dependent spatial memory may reflect the interaction between dopamine and estrogens. As only the groups that experienced changes in developmental conditions (LH and HL) were affected, timing of developmental stress arguably moderates the effects of stress on dopamine and estrogens.

6.4 CONCLUSIONS

Phenotypic plasticity is complex and multidimensional, influencing arrays of independent and interdependent traits at the molecular, systems, individual, and community level (Whitman & Agrawal 2009). The experiments in this dissertation highlight the importance of the timing of developmental stress (in the form of nutritional stress) in determining phenotypes, and show that developmental stress may have highly variable effects on certain traits. This implies that phenotypic variability may possibly arise from sex differences and individual differences in sensitivity to environmental cues. These findings would benefit from future investigations that examine timing of stress from an ecological context, such as whether the effects of change in developmental conditions on learning have fitness consequences, whether costs of phenotypic alterations are revealed in senescence, and the degree to which the effects of early compared to juvenile stress are transferable across generations.

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Ethics Approval

Dear Dr. **MacDougall-Shackleton**

Your Animal Use Protocol form entitled:

Stress, Development and the Avian Brain

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **09/01/10** to **08/31/11**

The protocol number for this project remains as **2007-089**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this *Animal Use Protocol* is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Curriculum Vitae

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Kriengwatana, B., Wada, H., Macmillan, A., MacDougall-Shackleton, S.A. (in press). Juvenile nutritional stress affects growth rate, adult organ mass, and innate immune function in zebra finches (Taeniopygia guttata). *Physiology & Biochemical Zoology.*

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Kriengwatana, B.P. (2009). Birdsong: Music to her ears and genes for her kids. Western Research Forum, University of Western Ontario, London, Ontario, Canada (contributed talk; received 2nd place and the Alumni Association Multidisciplinary Award).

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Block, A., Lieblich, S.E., **Kriengwatana, B.P**., Galea, L.A., FLoresco, S.B. (2006) Dose dependent biasing of strategy selection by estradiol in a set-shifting task. Society for Neuroscience Abstracts, Atlanta, GA.

Teaching experience:

Teaching assistant	Hormones and Behavior	2013
	Introduction to Behavioral and Cognitive Neurosciences	2012 - 2013
	Introduction to Cognition	2011
	Introduction to Personality	2011
	Honors Thesis	2008 - 2010
	Animal Behavior	2007
	Introduction to Psychology	2007
Guest lecturer	Introduction to Behavioral & Cognitive Neuroscience	2012, 2013
	Animal Behavior	2012
Undergraduate co-supervisor	James Brooymans-Quinn (Honors thesis, Psych)	2012 - 2013
	Sean Aitken (Honors thesis, Psych)	2010-2011
	Laura Garcia	2011
References:	Available upon request	