Effects of Early-Life Adversity on the Adult Phenotype in European Starlings

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Thesis Abstract

During development, individuals are exposed to a variable environment that shapes the adult phenotype. Circumstances that increase exposure to different sources of adversity during early life can significantly affect adult characteristics, extending so far as to contribute to the development of diseases such as depression and anxiety. Such psychological illnesses are the leading causes of disability worldwide, however there is still a significant amount yet to learn about the role that sources of early-life adversity can play in their etiology. Furthermore, even less is known about the mechanisms by which early experience becomes recorded in the adult phenotype. In a cohort of European starling nestlings, we used a unique developmental manipulation designed to dissociate effects of overall quantity of food from the begging investment required to obtain it. When the birds reached adulthood, we set out to investigate how early-life adversity could affect the HPA axis, DNA methylation, depression-like and anxiety-like phenotypes. We showed that different types of adversity can have significant independent effects on different components of HPA profiles, emotional phenotypes and global DNA methylation. We found evidence to suggest that increased exposure to adversity can decrease depression-like behaviour, but increase anxiety-like behaviour. We also showed that changes to the HPA axis are not stable as the birds age. We investigated if changes to the HPA axis were associated with depression-like and anxiety-like behaviour, but found limited evidence to support this hypothesis. Finally, we found that DNA methylation could be shaped by the early environment. We identified significant effects of nutritional restriction on global DNA methylation, with less food as a nestling leading to global DNA hyper-methylation. This work adds support to the hypothesis that different sources of early-life adversity can have significant effects on the adult phenotype.

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List of Abbreviations

5-mC - Methylcytosine

AICc - Akaike information criterion (corrected)

ASAB – Association for the study of animal behaviour

CEA – Cytosine extension assay

CORT - Corticosterone

CV – Coefficient of variation

DTA – Developmental telomere attrition

D_x – Days post-hatch where D_1 = day of hatch

ELISA – Enzyme-linked immunosorbent assay

GR – Glucocorticoid receptor

HPA – Hypothalamic-Pituitary-Adrenal

LE, LH, PE, PH – Combinations of the experimental treatment groups representing Lean-Easy, Lean-Hard, Plenty-Easy and Plenty-Hard birds respectively.

LME – Linear mixed effects model

LMPA – Low melting point agarose

LRT – Likelihood ratio test

LUMA – Luminometric assay

MeDIP – Methylated DNA immunoprecipitation

MR – Mineralocorticoid receptor

PCR – Polymerase chain reaction

RBC – Red blood cell

RIA – Radioimmunoassay

SAM – S-Adenosylmethionine

Chapter 1. Introduction

The study of the early-life environment is an ever-expanding topic in human and animal literature. During development, an individual is exposed to a highly variable environment that shapes the adult phenotype. Adverse circumstances that increase exposure to different sources of stress during early-life can have a drastic effect on adult characteristics, extending so far as to contribute to the development of diseases such as depression and anxiety. Such psychological illnesses are among the leading causes of disability worldwide, however there is still a significant amount to learn about the role that sources of early-life adversity can play in the etiology and development of these diseases. Further to this, far less is known about the mechanisms responsible for mediating the effect of the early-life environment on the adult phenotype.

This thesis will look at where, and how, different sources of early-life adversity can affect aspects of the adult phenotype in a long-lived avian species, using multidisciplinary physiological and behavioural techniques. Using a developmental manipulation designed to dissociate two different types of adversity, we will examine the effects of the juvenile environment on the stress response, anxiety-like and depressive-like behaviour, as well as investigating a suggested proximal mechanism (global DNA methylation) that could mediate phenotypic change resulting from exposure to the early environment. This chapter consists of a short literature review consolidating relevant work in this field, with more detailed introductions to each experiment provided in the following data chapters (chapters 3-6).

1.1 Studying Early-Life Adversity

Research on early-life adversity is prevalent in literature concerning a wide range of topics, such as human health, ethology, selection and adaptation. Early-life adversity can be defined as "the exposure to events during childhood that exceed coping resources and lead to prolonged periods of stress" (Pechtel & Pizzagalli 2011). Adverse circumstances can refer to both social (such as neglect, sexual abuse and witnessing violence) and physical stressors (such as poor nutrition, trauma or illness). Exposure to early-life adversity in humans has been correlated with many biological changes, such as altered gene expression, the development of abnormal adolescent or adult behaviour and alteration to physiological systems such as the stress response (McGowan et al. 2009; Heim et al. 2008; Anda et al. 2006). Adversity has also been associated with the etiology of several diseases such as depression and obesity (Sadowski et al. 1999; Noll et al. 2007). For example, Noll et al. (2007) showed that females

who had experienced sexual abuse as children were more likely to become obese later in life. From a biomedical perspective, study of the direct connection between childhood adversity and later effects is crucial through experimental manipulations, however this is not fully possible using human subjects. Adult consequences of exposure to different types of early-life adversity cannot be studied in a controlled and ethical manner in humans, meaning a complete picture of the effects of developmental adversity and the mechanisms that mediate them is yet to emerge.

Human studies of early-life adversity often rely on self-reported, retrospective measures of a broad range of social experiences, which are commonly summed into a single score for analysis. For some indices, children who have a parent with depression may be given the same score as a child who had experienced sexual abuse for over a decade. Inevitably, a large amount of information is omitted or uncontrolled in human studies, including the severity of the adversity, any current life stressors or past nutritional experience. Experiments involving animal models set out to combat this problem, developing controlled paradigms designed to manipulate early-life adversity, and to evaluate the effects of this in adulthood. In rodents, early-life adversity is often manipulated using dam-pup separation in the first two weeks after birth (Pryce et al. 2005). However, several problems exist with this design. First, different sources of adversity can be easily confounded by the manipulation itself (however see Crnic et al. (1981) for an exception). For example, adversity can be brought about through lack of grooming, lack of maternal protection, malnutrition or prolonged temperature changes. Second, due to the inherent difficulties of hand-raising rodents, these studies rely on dams rearing pups to adulthood. Unavoidably, this design introduces variation into the experience of early-life adversity through differences in the absolute quantity of food received and exposure to maternal hormones transferred through lactation. Many of these limitations can be overcome through the use of avian models.

1.2 Avian Models

Manipulating the early environment in bird populations is much simpler than in rodents, and examples of this have been documented in various protocols (e.g. Verhulst et al. (2006)). The use of an altricial bird species in experiments removes many of the difficulties involved in raising rodent pups, as young chicks have been shown to imprint on human caregivers with ease (Bateson & Asher 2010). Using birds in studies of early-life adversity allows experimenters the option to cross-foster eggs or chicks between nests and to subsequently hand-rear chicks once hatched. These advantages allow fine control when designing manipulations to alter the experience of early-life adversity. Cohorts of animals exposed to different types of adversity can be simply created and can remove confounds brought about by using dam-pup separation designs.

One such avian species used in the study of early-life adversity is the European starling (*Sturnus vulgaris*). European starlings are frequently used in experimental research and are currently one of the most commonly used wild animals in scientific studies (Asher & Bateson 2008). As a passerine species, their simple housing and husbandry requirements, naturally inquisitive behaviour, diurnal activity and small size make them ideal candidates for animal experiments (Bateson & Asher 2010). In many ways, the European starling acts as an analogous model to humans, and is valuable to the research of human systems. For example, it has been shown that avian endocrine pathways such as the stress response, or hypothalamic-pituitary-adrenal (HPA) axis, are very similar to mammalian systems and respond to stressors in an equivalent manner (Wingfield 2005). Starlings, similar to humans, are relatively long-lived (20+ years), produce altricial young that experience extensive juvenile periods and are highly social animals (Bateson & Feenders 2010). Rodents, however, have fairly short lifespans (2-3 years), and this can limit their use when making conclusions about enduring effects of early-life adversity in humans. Short-lived species (such as rats and mice) may not possess the same mechanisms as humans or passerine birds that allow for developmental flexibility to a wide variety of environments. Finally, wild-caught birds, as opposed to rodent stock bred from a long line of laboratory animals, allow us to study the natural phenotypic plasticity produced by different early-life situations. Experimental species such as rodents have reduced genetic variation, and may be adapted specifically to life within a laboratory environment.

1.3 Previous Work

Previous studies have shown that the European starling is indeed a viable model for the study of early-life adversity, finding both behavioural and physiological changes when birds were exposed to different levels of developmental stress. In 2012, cross-fostered starling siblings were placed in nests of either 2 or 7 chicks for 12 days post-hatch, simulating low and high levels of social competition respectively (manipulation described in Nettle et al. 2013). Whilst the brood size manipulation affected many characteristics such as dietary selectivity, growth and skeletal development (Bloxham et al. 2014; Nettle et al. 2013), Nettle et al. (2013) found that position of the bird in the weight hierarchy within the nest was a stronger predictor of early-life adversity experience than brood size. The bird's position in the hierarchy had a significant impact upon telomeres (non-coding DNA sequences that protect the ends of chromosomes and naturally shorten during ageing (Vaziri et al. 1993)). Having a greater number of heavier competitors in the nest accelerated developmental telomere attrition (DTA), a potential mechanism for linking environmental experience and the onset of age-related disease. Position in the weight hierarchy was also shown to predict adult affective (emotion-like) state, with birds who had faced fewer, heavier competitors in the nest shown to have

a higher expectation of reward when presented with a cognitive bias task (Bateson et al. 2015). However, manipulations that involve brood size adjustments are likely to have multiple sources of adversity that are impossible to tease apart, including both reducing the amount of food available as well as non-food consequences such as increasing begging effort experienced by a nestling.

To improve upon this and to manipulate competitive disadvantage directly (without varying the overall amount of food available to a nest), a new developmental manipulation was designed in 2013 in which chicks were placed in nests so that they were either the largest or smallest animals relative to other nestlings (manipulation described in Nettle et al. (2014)). Again, being at a competitive disadvantage was shown to increase rates of DTA (Nettle et al. 2014), as well as altering flight performance trade-offs and foraging behaviour (O'Hagan et al. 2015; Andrews et al. 2015). However, competitive disadvantage did not affect weight gain. As smaller nestlings have to beg more than larger siblings to ensure parental investment, these studies show that it is likely that increased begging effort can affect the adult phenotype, independent of nutritional shortages.

Whilst greatly adding to the study of early-life adversity, both the described manipulations have limitations. In both cohorts, birds were raised by starling parents in the field until post-hatch day 15 (2012) or day 12 (2013), introducing a large amount of uncontrolled parental and environmental variation during a critical period of starling development. The experimental designs are also unable to tease apart confounding effects of quantity of food (a physical stress) and begging effort (a social stress). With these manipulations, it is not possible to test the hypothesis that different sources of adversity can cause specific phenotypic outcomes. Therefore the need for a manipulation designed to dissociate these factors and manipulate them independently is crucial, and could add a great deal to the mechanistic literature relating to different sources of early-life adversity.

1.4 Mood Disorders

Alterations to stress-related physiology and the development of diseases such as mood disorders (when mood affects future unrelated decisions and situations) are areas in which early-life adversity is thought to play a significant role (Heim et al. 2000; Stein et al. 1996; Heim et al. 2008). Mood disorders are one of the world's leading causes of disability, with early-life adversity a proposed risk factor in the etiology of diseases such as major depressive disorder, post-traumatic stress disorder, mania and anxiety (Portegijs et al. 1996; Stein et al. 1996; Heim & Nemeroff 1999; Heim et al. 2008; Sadowski et al. 1999; Parker et al. 1995). Such diseases are widespread and usually recurrent, with sufferers often having a much reduced quality of life. In humans, vulnerability to developing these disorders include both genetic predispositions (sex, family history, personality) and environmental

experience (chronic stress, abuse, trauma) (Kendler et al. 2002; Nestler et al. 2002; Merikangas & Swendsen 1997). A female co-twin controlled analysis using twin pairs in which only one member had experienced early-life adversity (in this case, sexual abuse), showed that the exposed sister was more likely to develop a psychiatric illness, demonstrating a significant role of the early environment (Dinwiddie et al. 2000). Evidence suggests that early-life adversity can also affect mood in animals (Bateson et al. 2015; Overstreet 2012; Kalinichev et al. 2002; Caldji et al. 2000), however, the true extent which different sources of adversity can influence adult mood and affective state has not been investigated. Furthermore, the underlying physiology responsible for the development of mood disorders is not yet fully understood.

1.5 The HPA Axis

A large body of evidence shows that mood disorders could be partially explained by an alteration to the HPA axis (Weiler et al. 1982; Carroll et al. 1981; Vreeburg et al. 2010; Kallen et al. 2008; Condren et al. 2002; Erhardt et al. 2006; Holsboer 2000; Nemeroff CB & Vale WW. 2005; Warnick et al. 2009). To react to a stressful situation, defined as a state of real or perceived threat to homeostasis, animals elicit a wide variety of behavioural and physiological actions, known collectively as the stress response (Chrousos & Gold 1992). Ultimately, the stress response increases alertness, analgesia, heart rate, metabolism and cognition as well as reducing non-necessary immediate functions such as digestion, growth and immunity through the release of glucocorticoid hormones (Romero & Butler 2007). Glucocorticoids (corticosterone (CORT) found in birds, reptiles and amphibians and cortisol in most mammals and fish) regulate the physiological and behavioural changes seen in the stress response by acting on receptors distributed around the body, including the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Increased glucocorticoid levels initiate a negative feedback loop and function to inhibit and switch off the HPA axis (Keller-Wood & Dallman 1984).

The actions of the HPA axis and the release of glucocorticoids are beneficial, however excessive or prolonged activation of this response can be harmful, and it is thought that this can lead to the development of stress-related disease such as anxiety and depression (Arborelius et al. 1999; Abelson et al. 2007). For example, major depressive disorder is frequently associated with hyperactivity of the HPA axis through reduced negative feedback inhibition (Arborelius et al. 1999). The literature concerning the HPA axis and the development of anxiety disorders does not reach a consensus. Increased concentrations of glucocorticoid hormones are generally thought to promote anxiety-like behaviour (Fan et al. 2014; Mitra & Sapolsky 2008). However, many studies exist showing glucocorticoids to have anxiolytic properties (Albrecht et al. 2013; Heim & Nemeroff 2001). This is interesting as the human literature often reports comorbidity of anxiety and depression (Moffitt et al.

2009), suggesting, but not establishing, a common mechanism for the development of these disorders. It remains to be discovered if alterations to the HPA axis can act as a risk factor, a direct cause, or as a consequence of the development of different mood disorders.

Due to their suspected significant involvement in the development of depression and anxiety, factors that can cause alterations to the HPA axis (including exposure to early-life adversity) are of great interest to epidemiologists. There is a large amount of evidence showing that early-life adversity correlates with long-term changes to the stress response in humans. Heim et al. (2000) revealed that women previously exposed to childhood abuse had higher levels of adrenocorticotropic hormone (ACTH – a component of the HPA axis) when presented with a mildly stressful psychosocial situation of public speaking. Of these women, those diagnosed with depression had ACTH levels over 6 times greater than those of controls. Animal models of adversity have also shown that the early environment can have significant effects on the HPA response. In rodents, separation from the dam for 3-6 hours during the first 2 weeks of life leads to increased stressor reactivity and ACTH levels in adult rats when exposed to mild stressors (Plotsky & Meaney 1993). Ladd et al. (2004) went on to show that early-life separation in rats also reduces the expression of GR, leading to decreased inhibition of the stress response. It is clear that some sources of early-life adversity can have long-term and perhaps permanent effects upon the adult HPA axis, however the exact effects and their longevity, and the types of adversity that can affect the HPA axis, are not fully understood. It is not yet clear whether these effects are limited to changes in GR expression, circulating glucocorticoid concentrations, or if other components of the stress response are affected, such as the process of negative feedback inhibition.

1.6 Epigenetic Mechanisms

A further topic of interest in the study of early-life adversity is the proximal mechanisms that are involved in the shaping of the phenotype by the early environment. One such mechanism is epigenetics, defined as “long-term changes in gene function that do not involve a change in gene sequence or structure” (McGowan & Szyf 2010; Meaney & Szyf 2005). The most understood epigenetic process to date is DNA methylation. DNA methylation is the term given to biological change in DNA where cytosine bases are converted to 5-methylcytosine within the vertebrate genome by the addition of a methyl group. Increased DNA methylation in key exon enhancer or promoter regions is known to be associated with downregulated gene expression or even complete gene silencing (Jones et al. 1995; Jaenisch & Bird 2003; Berger et al. 2009), leading to subsequent changes in phenotype.

Changes in DNA methylation can be brought about by aspects of the environment, such as through exposure to tobacco smoke and pollution (Baccarelli et al. 2009; Zeilinger et al. 2013). Further to this, research has found that the sequence-independent epigenome goes through huge global and gene-specific *de novo* changes during development, indicating that this is a critical period of environmental vulnerability (Liang et al. 2011). Waterland & Jirtle (2003) were interested in discovering particular genetic loci that were affected by early nutrition in mice. They found that maternal dietary supplementation of folic acid, vitamin B_{12}, choline and betaine increased methylation of the *agouti* gene, responsible for producing melanocytes in mice, and also increased the number of offspring with pseudoagouti coat colours. DNA methylation is an excellent candidate to be explored as a mechanism for how the early environment can influence the adult phenotype, as it has been shown to be affected by different environmental exposures, is sensitive to significant change during development and can alter gene expression and phenotype. Studies that directly test how different aspects of the early environment (such as nutritional and social stressors) affect DNA methylation profiles can help identify the extent that epigenetic processes shape the phenotype.

Epigenetic changes have also been connected with exposure to early-life adversity. Methylation changes that occur in early life are of interest, as alteration to gene expression as a juvenile can have life-long health, physiological and behavioural effects. In humans, childhood abuse has been significantly correlated with changes to DNA methylation patterns both genome-wide (Essex et al. 2013) and at several specific loci, including at the serotonergic transmission gene (*5HTT*) and the GR gene (*NR3C1*) (Beach et al. 2010; Beach et al. 2011; McGowan et al. 2009). In rodents, Weaver et al. (2004) demonstrated that poor quality maternal behaviour could directly alter methylation status of the *NR3C1* exon 1_7 promoter, with hypermethylation seen in offspring who experienced less licking and grooming behaviour from dams.

Global DNA methylation has been fairly well described in mammalian species, including humans, rats and even baboons (Unterberger et al. 2009), however DNA methylation in birds has received much less focus in comparison. The first avian species in which global DNA methylation profiles were investigated across several tissue types was the chicken (Li et al. 2011). This found tissue methylation patterns to be largely similar to that of mammals and plants, demonstrating that conclusions gained from birds are likely to be representative of human systems. Studies that add to the literature concerning DNA methylation in bird species, as well as the effect of early-life adversity on DNA methylation across the genome are of significant value to the study of this topic.

1.7 Aims and Objectives

This thesis aimed to identify how different types of early-life adversity can affect adult behavioural and physiological phenotypes using the European starling as an animal model, and to consider the mechanisms that could shape this. To address this aim, we studied a cohort of hand-reared starlings that were subject to a developmental manipulation described in detail in chapter 2. The manipulation was designed to dissociate the effects of overall quantity of food received (Amount – Plenty or Lean) from the begging investment required to obtain it (Effort – Easy or Hard), thus creating 4 experimental treatment groups (Lean-Hard, Lean-Easy, Plenty-Hard, Plenty-Easy). Using the birds from this cohort once they reached adulthood, we aimed to identify physiological and behavioural measures relevant to the study of emotion and mood disorders that could be affected by early-life adversity. We measured corticosterone profiles, anxiety-like and depression-like behaviour independently, and aimed to investigate the relationship that corticosterone has with mood-related behaviour in this cohort. Finally, we aimed to evaluate epigenetics as a possible mechanism for mediating the effect of early-life environment on adult characteristics by measuring global DNA methylation in this cohort.

The thesis is structured as follows. Chapter 2 describes the developmental manipulation and cohort of animals used in this study and provides information concerning general methods used in this thesis. Chapter 3 presents the results of an experiment designed to measure the magnitude of the HPA response when the birds were 4 and 14 months of age, by taking measures of glucocorticoid concentrations after exposure to an acute stressor. We look at the effect that early-life adversity can have on the HPA axis and corticosterone profiles, and whether changes persist over time. Chapters 4 and 5 present the results of two behavioural studies that look at the effect of early-life adversity on anxiety-like and depression-like behaviour respectively in individual birds. These chapters consider the effect of early-life environment on affective state and the development of mood disorders, and also consider the relationship with physiological data collected in chapter 3. Chapter 6 examines a potential mechanism that has been suggested to be involved in mediating the effects of the early-life environment on later adult phenotype, by presenting the results of assays designed to measure levels of global DNA methylation. A relatively novel field of study, we go on to discuss the impact of measuring this in birds, and the implications that our methods could have on further studies of methylation. Finally, chapter 7 brings together these physiological, behavioural and mechanistic studies of environmental effects on adult phenotype, and discusses how this thesis contributes to the field of early-life adversity.

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Chapter 2. Materials and Methods

This chapter details general materials and methods that feature throughout this thesis. First, the developmental manipulation and animal subjects used in experiments in the following data chapters are described. The developmental manipulation was designed and conducted by members of the COMSTAR research group at Newcastle University (Daniel Nettle (DN), Melissa Bateson (MB) and Clare Andrews (CA)) prior to the start of this studentship (Nettle et al. 2017). Then, we discuss methodology common to all experimental chapters including blood sampling techniques, evaluating telomere length and attrition, body condition calculations and general statistical analyses. Telomere characteristics were analysed by Pat Monaghan and Sophie Reichert at the University of Glasgow at the end of the juvenile period. Experiment-specific methodology is outlined in the relevant data chapters (chapters 3-6). A timeline of the work presented in this thesis is shown in Figure 2.1. Finally, this chapter concludes by reviewing the immediate morphological and physiological outcomes of the developmental manipulation. These data was collected and analysed by DN, MB and CA, in collaboration with Carmen Martin-Ruiz and her team from the Newcastle biomarkers lab (Nettle et al. 2017).

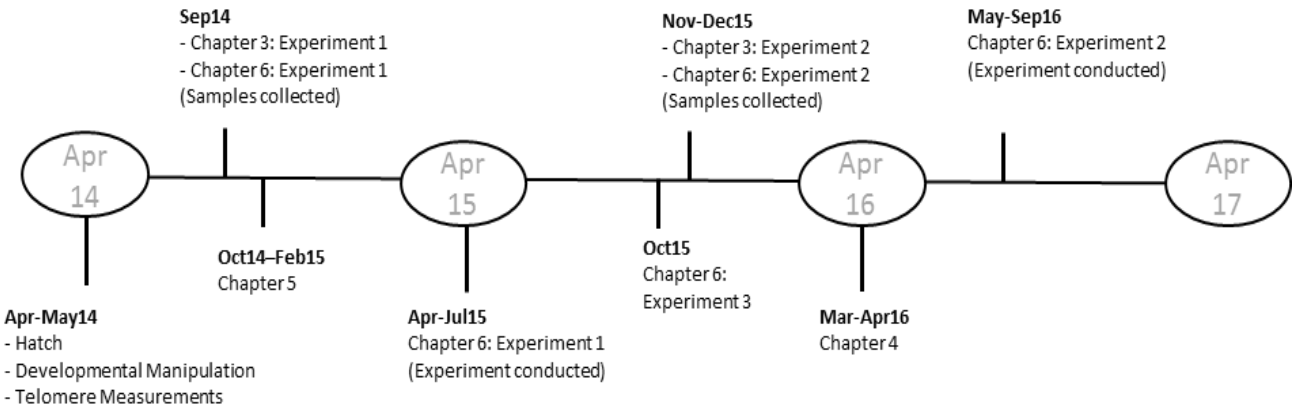

Figure 2.1: Timeline representing the key events and experiments referred to in this thesis.

2.1 Experimental Subjects

The European starlings (*Sturnus vulgaris*) referred to in this work all originate from a single cohort, hatched in spring 2014. The 32 birds were taken from monitored nests in Northumberland and consisted of four members, each from eight natal families. The chicks within each nest were likely to be full siblings as extra-pair fertilisations and brood parasitism occasions are rare within this species (Evans 1988). Chicks were taken exclusively from nests in which the eggs were uniform in colour and had accumulated at a rate of one per day (assessed by checking nest boxes daily). Birds were collected on the fifth day post-hatch (D5, where D1 = hatch) and upon arrival to the lab, one member from each family was randomly assigned to 4 experimental treatment groups containing 8 birds (Lean-Easy (LE), Lean-Hard (LH), Plenty-Easy (PE), Plenty-Hard (PH)). Each group was housed in two artificially covered nests of 4 chicks. There were no treatment differences in entry weight upon arrival into the laboratory (Nettle et al. 2017).

Sex of starling nestlings is not phenotypically observable so it was not possible to balance our experimental treatment groups with respect to sex upon entry to the laboratory. Instead, birds were genetically sexed post-manipulation to control statistically for sex in analyses. Molecular sexing was performed following a standard approach of amplification of the chromodomain-helicase-DNA binding (CHD) genes using real-time qPCR (Fridolfsson & Ellegren 1999). The cohort included 16 males and 16 females unevenly distributed across treatment groups (LE: 8M, 0F; LH: 3M, 4F; PE: 4M, 4F; PH: 1M, 7F).

2.2 Developmental Manipulation

The developmental manipulation took place between D5 and D15 and was designed to test the hypothesis that different kinds of early-life adversity can produce qualitatively different effects on the phenotype. We aimed to dissociate the effects of overall quantity of food received, from the begging effort required to obtain it. Briefly, two types of developmental experience were independently manipulated (Table 2.1). First, the amount of food provided (referred to in this thesis as an experimental treatment, Amount; either Plenty or Lean) and second, the perceived begging effort required to obtain this food (referred to as Effort; either Easy or Hard).

Table 2.1: 2x2 factorial design representing the four experimental groups. Eight sibling quartets were used, totalling 32 chicks (8 Plenty-Easy, 8 Lean-Easy, 8 Plenty-Hard, 8 Lean-Hard). The treatment groups included Plenty (ad lib food), Lean (approximately 75% ad lib), Easy (Food given on 9/9 occasions) and Hard (Food given on 9/18 occasions). The colours used to represent each experimental group are used throughout this thesis.

		'Amount' Manipulation	
		Plenty	**Lean**
'Effort' Manipulation	**Easy**	9 nest visits a day *Ad lib* food given every time	9 nest visits a day 73% of *ad lib* food given every time
	Hard	18 nest visits a day *Ad lib* food given at 9 nest visits	18 nest visits a day 73% of *ad lib* food given at 9 nest visits

Birds were hand-reared in experimental groups from D5 and all received nine feeds a day. Chicks were fed on a blended mixture of commercial poultry-based cat foods (Fit32, Royal Canin; Applaws Natural Cat Food Chicken Breast) mixed with apple sauce (A taste of Apple, Cow & Gate) and supplemented with vitamins (BSP drops, Vetark) and calcium (Zolcal D, Vertark), dispensed in 0.5ml aliquots, shown to be appropriate for hand-rearing starlings (Feenders & Bateson 2011). All nestlings were fed approximately every 90 minutes between 7:00am and 9:00pm. The proportion of *ad lib* fed to the Lean groups (described in the following section) was adjusted when necessary to maintain growth trajectories reported in nestlings from a previous study of wild-reared starlings (Nettle et al. 2013). Feeding sessions involved lifting the cardboard lid from the nest and dispensing food into the gapes of individual nestlings from a 25ml Eppendorf repeater pipette. The occurrence of begging bouts and the quantity of food delivered was recorded at each feed for every nestling.

2.2.1 Amount

The Amount treatment created Plenty and Lean experimental groups. During feeding bouts, Plenty groups were fed to satiation using a repeating pipette, defined as 5 seconds elapsing with an absence of natural begging or begging elicited when tapping the bill. Lean groups were given a pre-determined fraction of the mean intake of the corresponding Plenty group. On D6, this was 70% (recommended by Nowicki et al. 2002), however this was dynamically altered as the manipulation progressed to ensure chicks maintained lightest nestling growth trajectories as described above. Over the course of the manipulation, Lean birds received on average 73% of that of the Plenty groups.

2.2.2 Effort

The Effort treatment created Easy and Hard experimental groups. In addition to the 9 feeding bouts received by all birds, Hard birds received 9 'sham' feeds. Sham visits to the nest lasted two minutes

(similar time to a real visit in which food was delivered) and included the repeater pipette being waved above the chicks to elicit begging behaviour, however no food was delivered. Begging was recorded in all nestlings for at least part of the sham visit.

2.2.3 Post-Manipulation and General Husbandry

After D15, all birds were returned to sibling groups and fed age-appropriate *ad lib* food under identical conditions until independence (defined as fledging the bucket - approximately D21). Once independent, birds were group-housed in cages (75x45x45 cm) according to natal family. When self-feeding behaviour had been observed, birds were moved to indoor aviaries in groups of 20 (215x340x220 cm; 18^{o}C, 40% humidity) and had access to *ad lib* drinking water and *ad lib* food in the form of cat biscuits (Royal Canin Ltd. 'Fit'), domestic chick crumb (Special Diets Services 'Poultry Starter (HPS)') and dried insect paste (Orlux insect patée), supplemented with live mealworms and fruit. Enrichment was provided in the form of foraging substrate, water baths, multilevel rope perches and suspended cardboard boxes for cover. Birds were maintained in non-breeding circumstances by a constant 15:9h light:dark cycle at all times unless otherwise stated.

2.3 General Methodology

2.3.1 Blood Sampling and Cell Separation

Blood samples were required for analysing telomere attrition, corticosterone concentrations (chapter 3) and global DNA methylation profiles (chapter 6). For chicks, 70-120µl blood samples were taken from either the left or right alar vein, or a medial metatarsal vein using a 25 gauge needle and two 75µl heparinised microcapillary tubes on D5, D15 and D56. For adult bird samples, this limit was increased to 150µl. After sampling, birds were weighed and returned to the cage. Bleeding was stemmed under observation. Samples were immediately stored on ice at the collection point. Plasma and red blood cells (RBCs) were separated by centrifugation (3000rpm at 10 minutes) and frozen at -80^{o}C until required.

2.3.2 Telomere Attrition

RBC telomere length was measured in DNA from D5, D15 and D56 blood samples using a real-time PCR amplification method adapted for use in birds (Criscuolo et al. 2009; Cawthon 2002). Briefly, the telomeric sequence is expressed relative to a known single-copy gene (GADPH) producing a number (the T/S ratio calculated using the ΔΔCt method (Cawthon 2002)) representing relative mean telomere length for each sample (samples were assayed in triplicate). A reference curve to control for amplifying

efficiency was generated by serial dilutions of DNA standards on each plate. Amplification efficiencies calculated from the reference curves of the qPCR runs were between 107–112 (telomere) and 106–115 (control gene). R^2 calculated from the reference curves were minimum 0.98 both for the telomere and the control gene assays. Intra-plate mean coefficients of variation (CVs) for Ct values were 1.7% (telomere assay) and 0.4% (control gene assay). Inter-plate CVs for Ct values based on repeated samples were 2.7% (telomere assay) and 0.7% (control gene assay). T/S ratios calculated by incorporating variation in amplification efficiency (Pfaffl 2001) produced virtually identical results ($r>0.99$). For 5 birds (1 or 2 from each experimental group), the GAPDH assay failed and they were removed from further telomere analyses. To gain a single number of telomere shortening we used the D measure, correcting for regression to the mean (therefore 0 indicates the average amount of change in a sample and a negative number indicates more extreme telomere shortening) (Verhulst et al. 2013). Three D value measures (D5-56, D5-15 and D15-56) were calculated, with D5-56 used throughout this thesis as measure of developmental telomere attrition (DTA) over the complete juvenile period. DTA is thought to serve as a biological marker of the negative impact that early-life experience can have, and is used in this work as an alternative measure of adversity to experimental treatment group.

Developmental treatments were directly manipulated in this thesis, and tease out different types of adversity. The treatments capture the objective experience of each bird. DTA integrates all types of adversity (those we manipulated, and other unplanned sources) into a single measure, and accounts for the fact that different individuals may respond more or less strongly to a given objective circumstance. The strategy of the thesis is to present separate analyses of the two measures of early-life adversity, first with treatments as predictors, and then with DTA as the predictor.

2.3.3 Body Condition Calculations

Body condition indices in each experimental chapter (hereafter 'Body Condition') were calculated from body mass, correcting for tarsus length at D24 (mean of both tarsi at a time in which the tarsus is considered fully grown) using the equation (body condition index = mass – 2.92 * tarsus + 18.13). This was derived from a regression of mass gained between D115-123 (a period of housing in free-flight aviaries to reach a stable mass) on D24 tarsus length.

2.3.4 General Statistical Analyses

All data included in this thesis were analysed using the statistical programme R ("R Development Core Team" 2013) using both base statistical functions and general linear mixed effects models using package 'nlme' and 'lme4' (Pinheiro et al. 2017; Bates et al. 2015). All parameter estimates were by maximum likelihood (ML) and residuals were inspected for homogeneity and normality. If residuals

did not meet these criteria, the dependent variable was log transformed and the model fit retested. An alpha level of significance of $P < 0.05$ was used throughout (denoted by a *), however results of $P < 0.10$ (denoted by a ~) are also reported. Significance was assessed by a likelihood ratio test (LRT) on the difference in model deviance (Chi-square distribution) when a parameter was removed from the model setup. For each chapter, the data and R script are available online as supplementary material which can be accessed by either scanning or clicking on the following QR code.

Figure 2.2: QR code that if clicked or scanned allows access to an electronic copy of this thesis, relevant data files and R scripts.

In the following data chapters, the effect of potentially confounding variables that were not balanced between groups (body condition and sex throughout, and time taken to collect first blood sample in chapter 3) were assessed by testing each as a sole predictor on every dependent variable. If a significant effect was found, the covariate was retained in the main analysis. Throughout, fixed main effects of developmental treatment (Amount, Effort) and their interaction, and telomere attrition (DTA - an alternative measure of early-life experience) were tested against dependent variables referred to in each data chapter. In chapters 4 and 5, variables describing the stress response (described later in full - baseline CORT, peak CORT and ΔCORT (the difference between CORT measured at 15 and 30 minutes post-stressor)) were used as additional fixed predictors. Treatments, DTA and where applicable, corticosterone (CORT) variables were used as predictors in separate models (as opposed to one large mixed effects model) to increase statistical power and precision, and to give a more simple and informative interpretation of the results.

Interactions (denoted in this thesis by a * between predictor variables) and covariates were dropped from models if not significant to keep models as simple as possible. If significant interactions were detected, models were not simplified further. When interactions are indicated, they imply the presence of main effects. Random effects of natal nest and, where appropriate, individual bird were included to account for genetic effects, *in ovo*, very early environmental effects and repeated measures respectively. Models are reported alongside AICc (Akaike's adjusted information criterion,

a smaller-is-better index of model fit), LRT (likelihood ratio test), *P* values, parameter estimates (B ± SE) and n.

Corrections for multiple testing were not considered in this thesis, despite the use of multiple outcome measures in several data chapters. Cabin and Mitchell (2000) stress that the decision of when and how to apply corrections may be highly subjective, and is dependent on the individual hypotheses, experiments or questions that are to be considered. We argue that in this thesis, multiple testing corrections would be overly cautious. As much of the work presented is exploratory and novel, our method of analysis allows us to initially identify many aspects of adult behaviour and physiology that we believe can be significantly affected by the developmental treatments. The creation of such a novel cohort means that it is not possible to predetermine expected effect sizes. Using the information in this thesis, it is then possible to design experiments to be repeated in this cohort of animals with a reduced number of outcome measures that removes the need to correct for multiple testing.

The creation of this unique cohort was highly time consuming and is unlikely to be easily repeated. Therefore, the cost of missing significant effects (Type II error) due to multiple testing corrections is great, whereas Type I errors can be identified through repetition. In this cohort, we detect significant results at a level greater than 5% chance (seen in experimental results presented in the data chapters), alongside other unpublished work conducted using this cohort of animals. Therefore, it is likely that many of these effects are true positives. Despite this, we are cautious of our interpretations of significant results, stressing the limitations of small sample sizes and experimental order effects, where appropriate. We do not comment on the significance of results that approach significance to avoid making false inferences ($0.05 < P < 0.1$).

2.4 Ethical Considerations

Birds were collected under Natural England permit 20121066 and collection was approved by the Animal Welfare and Ethics Board at Newcastle University and the UK Home Office (licence PPL 70/8089). The manipulation and all studies described were carried out in accordance with the Home Office licence and the Association for the Study of Animal Behaviour (ASAB) guidelines for the use of animals in research. Fieldwork was carried out with landowners' permission with nest disturbances minimised. All nestlings brought into the laboratory gained weight rapidly after arrival indicating fast recovery from transportation and acceptance of hand-feeding (Nettle et al. 2017). Birds were marked with coloured tape around their tarsi until large enough to be given coloured leg rings, with no adverse effects of either method. The developmental manipulation was intended to increase stress experienced in some experimental groups, but were likely to have improved the experience of some

nestlings relative to wild animals, as mortality rates are naturally high in starlings (Feare 1984). We experienced no nestling mortality and all birds fledged successfully. Two birds (1 Lean-Easy, 1 Lean-Hard) died prior to 20 months.

Several studies reported in this thesis required analysis of blood samples. For European starlings, the maximum allowed blood sample volume is 10% total blood volume (on average 70ml/kg). When taking three samples, this equated to the maximum sample volume allowed per sample = 163µl. In adults, we aimed to take samples of 120µl, well within the legal home office limit and total number and volume of all samples taken were recorded and tracked.

2.5 Developmental Manipulation Outcomes

Here, I will review some of the outcomes of the developmental manipulation relating to weight, skeletal growth and telomere attrition that are of interest to work contained in this thesis. For a full review of the effects of the 2014 developmental manipulation, please see Nettle et al. (2017).

2.5.1 Telomere Attrition

Telomeres shortened over the course of early life, measured as the difference between telomere length at D5 and D56 (Paired T-test $t_{26} = -5.76$, $P <$ **0.01***) with the majority of this shortening occurring between D5 and D15 (Figure 2.2a). There were significant main effects of both Amount and Effort on DTA, with Lean birds and Hard bird groups associated with greater telomere attrition than Plenty or Easy birds respectively. There was no interaction seen between the treatments (Table 2.2, Model 1-2; Figure 2.2b). The group with the greatest DTA overall was Lean-Hard, with Plenty-Easy birds showing less DTA.

Table 2.2: Output from linear mixed effect models predicting developmental telomere attrition (DTA) between D5 and D56 from experimental treatments (Amount, Effort). A * represents an interaction term.

Model Number	AICc	Outcome Variable	Fixed Predictors	LRT	P	B (± SE)	N
1	4.4	DTA (D5-56)	Amount*Effort	2.31	0.13	-0.20 (0.12)	27
2	2.5	DTA (D5-56)	**Amount**	7.14	**< 0.01***	0.20 (0.07)	27
			Effort	5.19	**0.02***	-0.16 (0.07)	

2.5.2 Body Size and Skeletal Growth

Over the course of the developmental manipulation, there was a significant effect of both treatments on weight gain, with Lean-Hard birds gaining weight at the slowest rate (Table 2.3, Model 3; Figure 2.3a). At both D15 (the end of the developmental manipulation) and D56, tarsus length was affected by Amount, but not Effort (Table 2.3, Model 4-7; Figure 2.3b) with Lean birds remaining skeletally smaller throughout early-life. Age at fledging was also affected by the treatment groups, with Lean-Hard birds fledging significantly later than other groups (Table 2.3, Model 8; Figure 2.3c). At D56, a difference in weight due to both treatments was still detectable, with Lean-Easy birds being significantly heavier than all other experimental groups, and the Lean-Hard birds remaining smallest overall (Table 2.3, Model 9; Figure 2.3d).

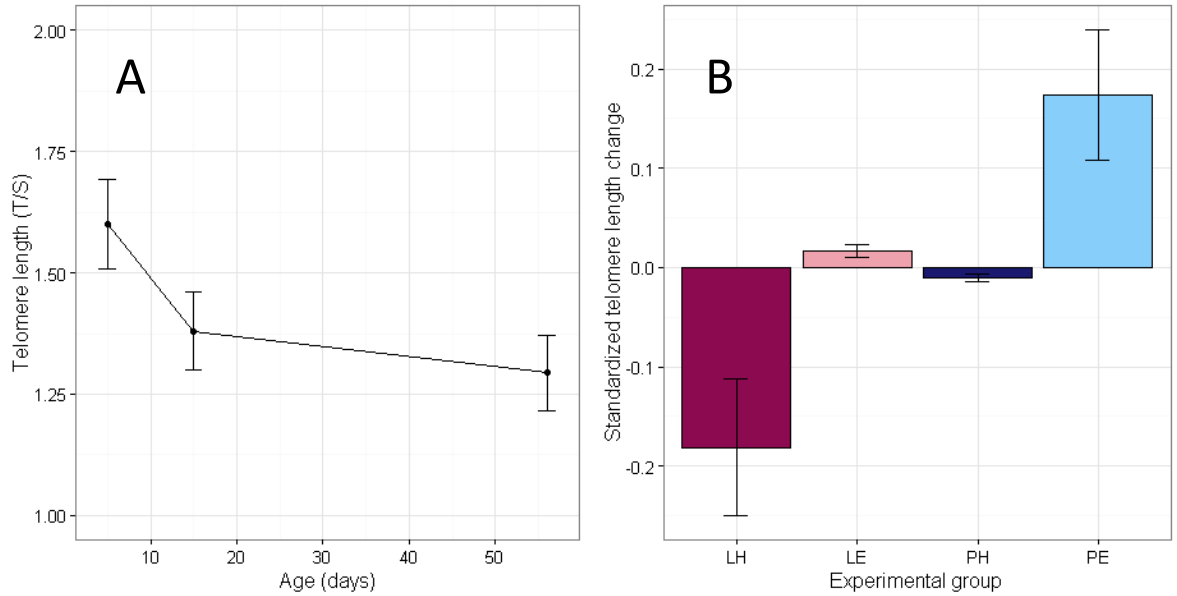

Figure 2.3: A) Mean telomere lengths at D5, D15 and D56 (n=27). Error bars represent one between-bird SE. B) Standardised DTA grouped by experimental treatment group over the entire developmental period (D5-D56) Error bars represent 1 SE.

Table 2.3: Output from linear mixed effect models predicting morphological developmental manipulation outcomes from experiment treatments (Amount, Effort). Where abbreviated, A = Amount, E = Effort. A * represents an interaction term.

Model Number	AICc	Outcome Variable	Fixed Predictors	LRT	P	B (± SE)	N
3	1844.4	Weight Gain	**A*E*Age**	7.04	**< 0.01***	0.54 (0.20)	32
4	83.0	Tarsus length (D15)	Amount*Effort	2.91	0.09~	0.80 (0.45)	32
5	82.6	Tarsus length (D15)	**Amount** Effort	24.79 0.02	**< 0.01*** 0.89	1.66 (0.27) 0.04 (0.28)	32
6	77.9	Tarsus length (D56)	Amount*Effort	2.76	0.10~	0.72 (0.42)	32
7	77.4	Tarsus length (D56)	**Amount** Effort	18.22 0.21	**< 0.01*** 0.64	1.24 (0.24) 0.12 (0.26)	32
8	77.7	Fledging	**Amount*Effort**	6.69	**< 0.01***	-1.19 (0.42)	32
9	170.2	Weight (D56)	**Amount*Effort** **Tarsus (D56)**	7.52 3.90	**< 0.01*** **0.05***	4.61 (1.57) 1.52 (0.68)	32

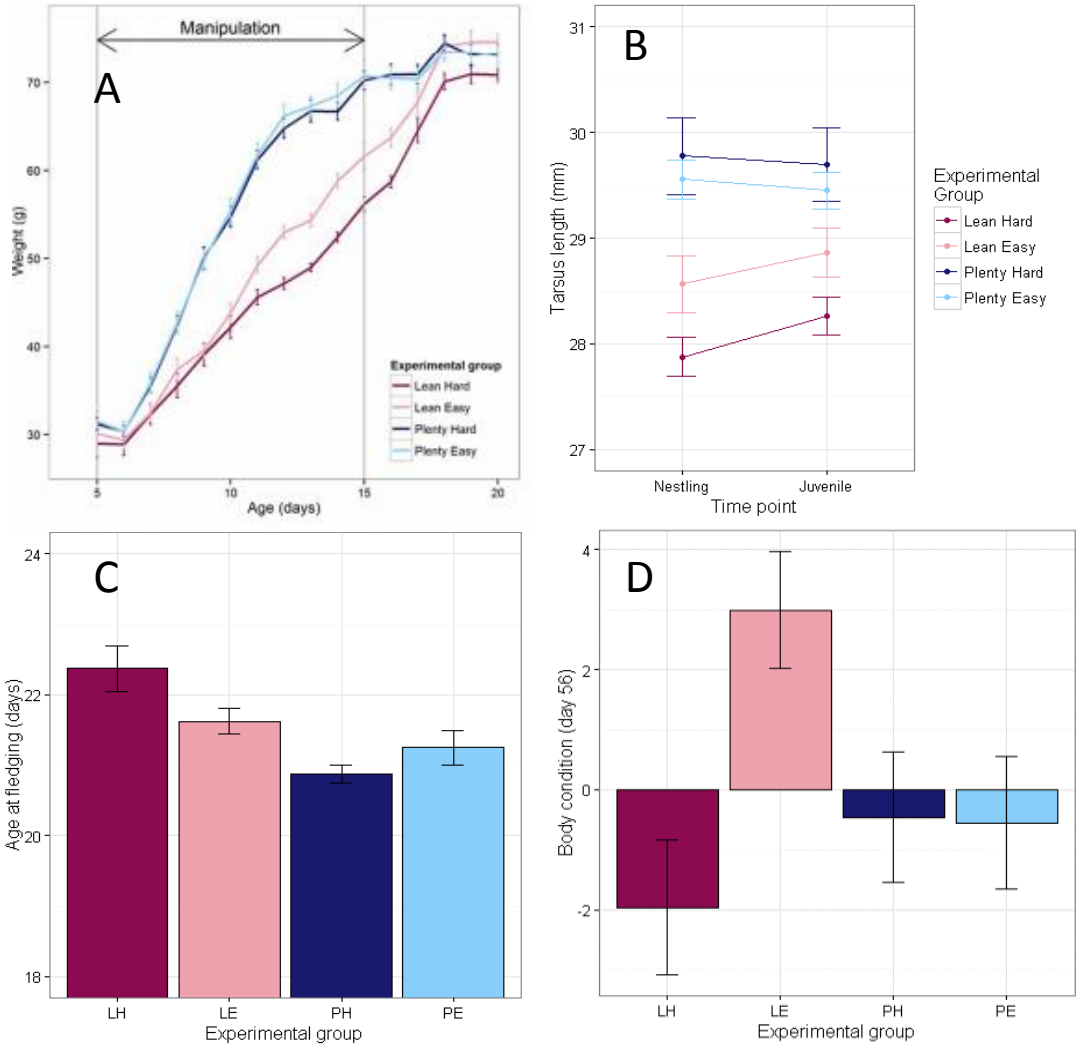

Figure 2.4: A) Mean daily weight grouped by experimental group during and immediately after the developmental manipulation. B) Mean tarsus length by experimental group. Here, nestlings represents measurements taken at D15 and juveniles as measurements taken at D56. C) Mean age of fledging (days) by experimental group. D) Body condition (Weight relative to skeletal size) by experimental group on D56.

2.6 Discussion of the Manipulation

The manipulation described above had significant effects on various aspects of juvenile morphology, and allowed us to separate out different sources of adversity, namely quantity of food received as a juvenile (Amount) and begging effort required to obtain this food (Effort). Lean-Hard birds (those who had experienced the most adversity) had more DTA, slower weight gain, were in the poorest body condition and were last to fledge compared to other developmental treatment groups. Lean birds were skeletally smaller than Plenty birds, however there were no significant differences between Hard and Easy birds. The direct outcomes of the manipulation showed both aspects of adversity can significantly affect juvenile traits, in both additive and interactive ways. For example, Amount and Effort had significant independent effects on DTA, however there was no interaction detected between the different sources of adversity. For other characteristics such as weight gain and age of fledging, Amount and Effort showed a significant interactive effect. These data imply that both physical and social stressors have a negative impact in bird species. Interestingly, other measures were affected by only one treatment. Skeletal size was shown to be affected by Amount but not Effort, highlighting the importance of separating out different sources of early-life adversity during manipulations. Finally, the data produced by the developmental manipulation described in Nettle et al. (2017) do not give indications of how long these effects persist into adulthood, and what, if any, effects they have on the adult physiological and behavioural phenotype.

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Chapter 3. Stress Physiology and the HPA Axis

Abstract

Exposure to early-life adversity is a known source of alterations to physiological systems such as the hypothalamic-pituitary-adrenal (HPA) axis. In response to a perceived threat, the HPA axis elicits a stress response by secretion of glucocorticoid hormones, such as corticosterone (CORT). Correlational studies have shown that childhood adversity and chronic activation of the HPA axis are associated with permanent alterations to the adult stress response. In turn, these alterations are thought to be involved in the etiology of mood disorders such as depression and anxiety. Studies using animal models have experimentally tested the hypothesis that early-life adversity can alter the adult stress response. In this study, we asked how two natural forms of early-life adversity could affect the adult HPA axis, and if these results were long-lasting. In a cohort of hand-reared European starlings (*Sturnus vulgaris*), a two-by-two factorial design was used to simulate different levels of early-life adversity by separately manipulating food availability (Amount – Plenty vs Lean) and begging effort (Effort – Easy vs Hard) in the first two weeks post-hatch. We examined the strength of the HPA axis in response to an acute capture-handling-restraint stressor, measuring baseline CORT, peak CORT and the HPA negative feedback process (ΔCORT), at 4 and 18 months of age. We found evidence to suggest that developmental treatments had both significant additive and interactive effects on the HPA axis, but specific effects differed between time points. At 4 months of age, the data suggested that increased begging effort could lead to the development of an attenuated stress response, with most efficient responses seen in birds from Hard treatment groups. Specifically, Plenty-Hard birds were shown to have lower levels of peak CORT, exposing the complicated interaction between different sources of adversity. At 18 months of age, treatment effects on peak CORT were no longer detectable, however an Amount by Effort interaction remained on ΔCORT. Here, peak CORT in Plenty-Easy birds fell fastest to baseline conditions, whereas CORT levels continued to rise in Plenty-Hard birds. We concluded that different components of the HPA axis can be significantly affected by separate sources of early-life adversity into adulthood, and that effects are not necessarily stable over time.

3.1 Introduction

Exposure to early-life adversity is known to have long-term effects on physiological systems such as the hypothalamic-pituitary-adrenal (HPA) axis (Neal et al. 2004; Glover et al. 2010; Morley-Fletcher et al. 2003). The HPA axis elicits a stress response, functioning to promote survival when an animal is faced with an acute threat. Effectiveness of the stress response is commonly measured by secretion of glucocorticoid hormones (corticosterone (CORT) found in birds, reptiles and amphibians and cortisol in most mammals and fish). Release of CORT increases the availability of glucose, diverts blood flow to essential areas (such as skeletal muscles) and restricts unnecessary functions (such as digestion) (Romero & Butler 2007). Commonly, studies measure the strength of the stress response by quantifying baseline CORT (the concentration of the hormone continuously secreted without exposure to a stressor) and peak CORT (the increased concentration of CORT after a stimulus is perceived to be a threat). Some studies also take into account the ability of the HPA axis to terminate the stress response, through the process of negative feedback (measured as ΔCORT – typically the change in CORT between two time points after the stressor has terminated). This is mediated by the binding of CORT to glucocorticoid (GR) receptors. The HPA axis is beneficial in response to acute stressors, however can be permanently altered when exposed to periods of chronic stress. This, in turn, can leave an individual susceptible to disease (Mizoguchi et al. 2001; Anisman et al. 1998; Weaver et al. 2000).

A critical period of chronic stress exposure is during early life. Correlational studies have shown that the prevalence of depression is increased in humans that have suffered from various forms of early-life adversity such as sexual abuse or maltreatment (Kendall-Tackett 2002; Sachs-Ericsson et al. 2007). However, the mechanisms responsible for the development of such diseases are not able to be demonstrated in humans. Faravelli et al. (2010) hypothesised that when the HPA axis is hyperactive or chronically stimulated during childhood, it can become permanently altered, possibly through disrupted function of GR (Anacker et al. 2011). Heim et al. (2008) demonstrated this effect, finding that men who had been abused as children had an increased stress-induced cortisol response, and suggested that childhood adversity is associated with impaired glucocorticoid feedback control of the HPA axis. Further to this, several studies have demonstrated that disruption to the negative feedback process of the HPA axis is present in approximately half of humans suffering from depression (Weiler et al. 1982; Carroll et al. 1981). Animal models provide a way of investigating the links between early-life adversity, impaired HPA function and the development of adult disease.

Studies using mammalian species have shown significant effects of post-natal stress and experimental increase of glucocorticoid exposure on the stress response and adult behaviour (Parfitt et al. 2007;

Neal et al. 2004). Perhaps most famously, studies in rodents have shown that pups who experience high maternal care exhibit increased negative feedback and an attenuated stress response (Liu et al. 1997). However, the process of lactation confounds the effects of maternal glucocorticoid hormone transfer and direct alterations to the offspring HPA axis (Spencer et al. 2009). Studying altricial birds instead can overcome this problem, as birds have been shown to maintain a similar degree of post-natal HPA axis development as seen in rodents (Schwabl 1999).

In birds, many conditions experienced within a nest have been shown to increase CORT secretion, for example, competition from siblings, parasitism, parental absence and nutritional shortages (Vallarino et al. 2006; Raouf et al. 2006; Rensel et al. 2010; Pravosudov & Kitaysky 2006). The majority of studies, however, do not follow exposed individuals into adulthood to assess long-term effects. Spencer et al. (2009) first showed that direct dietary administration of CORT in early-life can lead to long-term changes to HPA function in zebra finches (*Taeniopygia guttata*). When tested as adults, birds who had been CORT-treated had reduced negative feedback capability when compared to controls, but no difference in baseline CORT concentrations. Whilst useful, this does not represent conditions that a bird would naturally experience in the wild. Brood manipulations have given insight into this. Saino et al. (2003) altered brood size and food availability to barn swallows (*Hirundo rustica*) and showed that nestlings from enlarged broods and from those who had limited food access had higher baseline CORT concentrations. Enlarging broods, however, is likely to have several confounded effects, including reducing absolute food quantities whilst simultaneously increasing begging behaviour and social competition (Nettle et al. 2017). Additionally, the long-term effects of this manipulation were not studied. Pravosudov & Kitaysky (2006) hand-reared Florida scrub-jays (*Apheloocoma coerulescens*), feeding one group to satiation and another approximately 65% of this amount between day 7 and day 30 post-hatch. Whilst noting significant differences in baseline CORT during exposure to the treatments, there were no differences remaining at 12 months. However, at 12 months of age, nutritionally deprived jays had significantly higher peak CORT concentrations. This study stresses the importance of investigating the longevity of early-life effects on CORT profiles in adults.

CORT profiles are known to differ between individuals of the same species (Wingfield et al. 1994), however within-individual variation (or repeatability) of CORT responses over time is important to consider, as low individual repeatability could affect interpretation of these differences (Romero & Reed 2008). The literature surrounding repeatability of baseline CORT and peak CORT concentrations within an individual provides mixed results. Whilst there are several studies that demonstrate the repeatability of CORT profiles in a variety of avian species (Cockrem & Silverin 2002; Kralj-Fišer et al. 2007; Wada et al. 2008; Angelier et al. 2010), it is common to find examples in which repeatability is either limited, or context-specific (Lendvai et al. 2015; Rensel & Schoech 2011; Ouyang et al. 2011;

Baugh et al. 2014; Small & Schoech 2015; Romero & Reed 2008). Rensel & Schoech (2011) comment that repeatability of (baseline) CORT measures are difficult to ascertain in wild populations, without the ability to control recent experience that may affect an individual's stress response. Variation in environmental conditions (both adult and developmental) can have a dramatic impact upon the stress response, and highlights the importance of studies in which both juvenile and current experience can be controlled. It is, as yet, unknown if CORT measures (baseline CORT, peak CORT and ΔCORT) are repeatable when tested in a laboratory-raised population of birds.

This study aims to investigate the relationship between early-life adversity and the adult stress response in a long-lived passerine species, the European starling (*Sturnus vulgaris*). In a cohort of hand-reared birds, a two-by-two factorial design was used to simulate different levels of early-life adversity by separately manipulating food availability and begging effort in the first two weeks post-hatch (Nettle et al. 2017; Chapter 2). At the end of the manipulation, birds were raised in uniform conditions until the time of the adult stress response experiments. Developmental telomere attrition (DTA), a biomarker of biological age, was measured in erythrocytes and was found to be accelerated in birds who had experienced the lowest amount of food and the highest begging effort (Nettle et al. 2017; Chapter 2). Our hand-rearing design allows the separate effects of nutritional shortages and begging effort to be explored. The design also removes the influence of any uncontrolled sources of variation not part of the experiment, experienced during development or further into adulthood.

Here, we report on baseline CORT, peak CORT and a measure reflective of the HPA negative feedback process (ΔCORT) in adult European starlings from the 2014 cohort when exposed to an acute capture-handling-restraint stressor at two time points, 14 months apart (when birds were approximately 4 and 18 months of age). We predicted that birds exposed to more adverse treatments (and ultimately had more DTA) would show a greater overall stress response with higher levels of peak CORT and slower recovery towards baseline CORT at both experimental time points. We did not expect to see treatment differences in baseline CORT as previous literature show that treatment-induced baseline CORT effects dissipate over time (Spencer et al. 2009; Pravosudov & Kitaysky 2006). Finally, we predicted that there would be some repeatability between baseline and peak CORT levels within individual birds. Negative feedback repeatability, to our knowledge, has not been conducted in any species, however we predicted that this would also show individual consistency when measured at two time points.

3.2 Materials and Methods

3.2.1 Note on Experiment 1 and Experiment 2

This study consists of two separate stress response measurements taken from the same individuals at different time points (Experiment 1 and Experiment 2). Data for Experiment 1 were collected by CA and analysed by AG when birds were 4 months old. Data for Experiment 2 were both collected and analysed by AG when the birds were 18 months old. In both experiments, the methodology was identical unless otherwise specified.

3.2.2 Housing and Husbandry

Subjects in this study were adult European starlings that had been subjected to a unique developmental manipulation described previously (Chapter 2; Nettle et al. 2017), designed to dissociate the effects of overall quantity of food received (Amount – Plenty or Lean) from the begging investment required to obtain it (Effort – Easy or Hard). Experiment 1 consisted of 32 birds aged 127-134 days (16M, 16F; 8LH, 8LE, 8PH, 8PE). Experiment 2 consisted of 30 of the same birds aged 584-601 days (15M, 15F; 7LH, 7LE, 8PH, 8PE – 2 birds had died between experiments).

For the stress response experiments, two experimental rooms were used, each housing groups of up to eight birds per room. Experiment 1 was conducted in two batches over a two week time period. Experiment 2 was conducted in three batches over a three week time period. Birds were weighed upon entry to the experiment and again at time of sampling to gain a measure of body mass and body condition. This was to assess treatment differences, and also as energetic balance is thought to play a role in HPA function (Love & Williams 2008). Birds were individually caged (75x45x45cm) whilst maintaining full acoustic and visual contact with others for a period of 3-27 days (Experiment 1 mean 5 days, Experiment 2 mean 10 days). All animals had access to two wooden perches, two drinking bottles, a water bath (removed approximately four hours prior to stress series sampling) and a bowl containing *ad libitum* food (10g cat biscuits, 5g turkey crumb, 5g Orlux and 1/8 of a piece of fruit replaced daily). The birds were maintained in environmental conditions identical to the free-flight aviaries. Cage position (upper and lower) were counter-balanced with respect to developmental treatment. Habituation to the cages occurred over a minimum of three nights prior to blood sampling. All birds were retained at Newcastle University for further experiments.

3.2.3 Stress Response Measurements

We used a standardised capture-handling-restraint protocol (Wingfield et al. 1994) known to induce an acute stress response to measure both baseline and peak CORT concentrations in blood plasma. Stress response sampling was carried out during a period of minimal diurnal CORT variation (Romero & Remage-Healey 2000). In experiment 1, this time period was between 1330 and 1500. In experiment 2, for 23 birds, this time period was between 1400 and 1500. For 7 birds, who had been time-shifted for a separate behavioural experiment, this time period was between 1630 and 1730. Therefore, all animals were sampled at the same biological point in the circadian cycle (with minimal diurnal CORT variation). Daily, we measured an acute stress response of a minimum of two randomly selected birds.

Birds had not been disturbed for 2 hours prior to sampling. The acute stressor began immediately after the lights were switched off in the experimental room and two experimenters entered the room (Time 0). Using torches as an aid, birds were caught from the cages and transferred immediately to an adjacent room used for blood sampling (full methodology described in chapter 2). Approximately 150µl of blood was collected as a baseline CORT sample as quickly as possible from the left alar vein. All initial samples were taken within three minutes of first entry to the experimental room (experiment 1: mean time to baseline sample ± SD, 94.9 ± 23.6s; experiment 2: mean time to baseline sample ± SD, 108.4 ± 29.7s). One bird (female, PH) was removed from analysis in experiment 1 as time to sample was at the upper limit (180 seconds) and visual analysis of the radioimmunoassay data confirmed a very high level of baseline CORT. This bird was removed from subsequent analyses (experiment 1: resultant mean time to baseline sample ± SD, 92.1 ± 18.1s). Bleeding was stemmed using cotton wool and birds were placed in drawstring cloth bags until 15 minutes after the initial disturbance (Time 15). A second 150µl blood sample was collected from the right alar vein and the bird was then returned to the cloth bag for a further 15 minutes. Thirty minutes after the initial disturbance (Time 30), a final 150µl blood sample was taken from a medial metatarsal vein. Birds were then weighed and returned to the cage under observation. Daily husbandry was conducted approximately 30 minutes after sampling completion and cages containing sampled birds were checked for blood.

Blood samples were transferred to a labelled Eppendorf tube and placed on ice immediately until centrifugation (< 60 minutes). Following collection of the third blood sample, tubes were centrifuged (10 min at 3000 rpm) to separate plasma from erythrocytes. Plasma was isolated using a Gilson pipette and stored at -80^{o}C until radioimmunoassay analysis. Three birds in experiment 1 and six birds in experiment 2 were repeat-sampled after a minimum of 24 hours due to either insufficient blood volume collected, or samples being collected outside of the time constraints needed for collecting a baseline sample. Samples were all of a sufficient volume, with 31 birds in experiment 1 (1 bird

excluded) and 30 birds in experiment 2 having three CORT samples representing a complete stress response.

3.2.4 Corticosterone Radioimmunoassay (RIA)

CORT levels in plasma extracts were quantified using radioimmunoassay (RIA) previously validated in European starlings (Buchanan et al. 2003). CORT concentrations from the three time points were measured after extraction of up to 35µl aliquots of plasma in 1ml ethyl diether by a Dextran-coated charcoal RIA method (Wingfield et al. 1994) with [1,2,6,7-3H]-CORT using an anti-CORT serum ABIN880 (Antibodies Online). The cross reactivity data for ABIN880 were: deoxycorticosterone 1.5%, cortisol <0.1%, aldosterone 0.2% and progesterone <0.1%. All CORT levels were above the detection limit (0.04 $ngml^{-1}$). For all samples, extraction efficiency was estimated at between 77-100% (mean 96.1%), with final CORT concentration values being corrected accordingly. Samples were run in duplicate in a single assay (intra-assay coefficient of variation 14%).

3.2.5 Statistical Analysis

The stress response was characterised by three dependent variables: a) Baseline CORT concentration (taken at Time 0); b) Peak CORT concentration (taken from the higher of Time 15 and Time 30 samples); c) ΔCORT - the change in CORT between Time 15 and Time 30 samples (calculated as CORT Time 15 – CORT Time 30; ΔCORT is a positive value when CORT concentrations decrease between Time 15 and Time 30, and a negative value when CORT continues to increase). All birds showed lowest levels of CORT at baseline when compared to concentrations at Time 15 and Time 30. Peak CORT was used rather than CORT at Time 15, as the timing of maximum CORT output varied between Time 15 and Time 30. There were no significant differences between the timing of peak CORT (Time 15 or Time 30) accountable to treatment group in either experiment (experiment 1: $X^2 = 2.38$, $P = 0.50$ n = 31; experiment 2: $X^2 = 2.66$, $P = 0.45$, n = 30). We also calculated Total CORT output (calculated as area under the response curve), but this was excluded from further analysis due to a strong positive correlation with peak CORT concentrations in both experiments (experiment 1: r = 0.90, $P <$ **0.01***; experiment 2: r =0.91, $P <$ **0.01***).

3.2.5a Analysis of Each Experiment Separately

The effect of potentially confounding variables that were not balanced between treatment groups (body condition, sex and the time taken to collect the first blood sample) were assessed by testing each as a sole predictor on each CORT variable. In experiment 1, body condition and sex had a significant effect on peak CORT concentrations and were used as covariates throughout analyses of

this variable. In experiment 2, none of the extraneous variables were significant and the variables were not included in the main analyses. Throughout, where the outcome variable was peak CORT, baseline CORT was used as a covariate and where the outcome variable was ΔCORT, CORT concentration at Time 15 was also included as a covariate (as individuals with higher CORT at Time 15 were more likely to have reached the peak of their CORT production and have begun to return to baseline concentrations). Where analysing the effect of developmental treatments, the base model for each CORT variable had fixed effects of Amount, Effort as well as the interaction between the two treatments. Where analysing the effect of DTA, the base model included a fixed effect of DTA between day 5 and day 56 post-hatch. A random effect of natal nest was used throughout to control for non-independence of birds from the same family.

3.2.5b Combined Analysis

To compare the two experiments, we combined the data and retested the effects of confounding variables. In the combined dataset, body condition significantly affected peak CORT and was retained in analyses of this variable. Time taken to collect the first sample had a significant effect on combined baseline CORT and was also retained. When analysing the effect of developmental treatments, the base model had fixed effects of Amount, Effort and experiment (1 or 2), with the interactions between the variables. Where analysing the effect of DTA, the base model had fixed effects of DTA between day 5 and day 56, experiment, and the interaction between them. Throughout, random effects of natal nest and individual bird were used to control for repeated measures. We directly examined the repeatability of baseline CORT, peak CORT and ΔCORT from data collected in experiment 1 and experiment 2 using intra-class correlation coefficients using the equation described by Lessells & Boag (1987).

3.3 Results

3.3.1 Experiment 1

The stress response measurements presented in this section refer to the first stress series conducted when the birds were 4 months old.

3.3.1a Sex and Body Condition

Mass and body condition were significantly correlated (r = 0.97, $P <$ **0.01***). Males had a significantly higher body condition than females (mean males 7.11, mean females 1.31, Table 3.1) and there was also a significant interactive effect of the developmental treatments on body condition (mean LE 7.89, mean LH 1.07, mean PE 4.59, mean PH 3.55, Table 3.1).

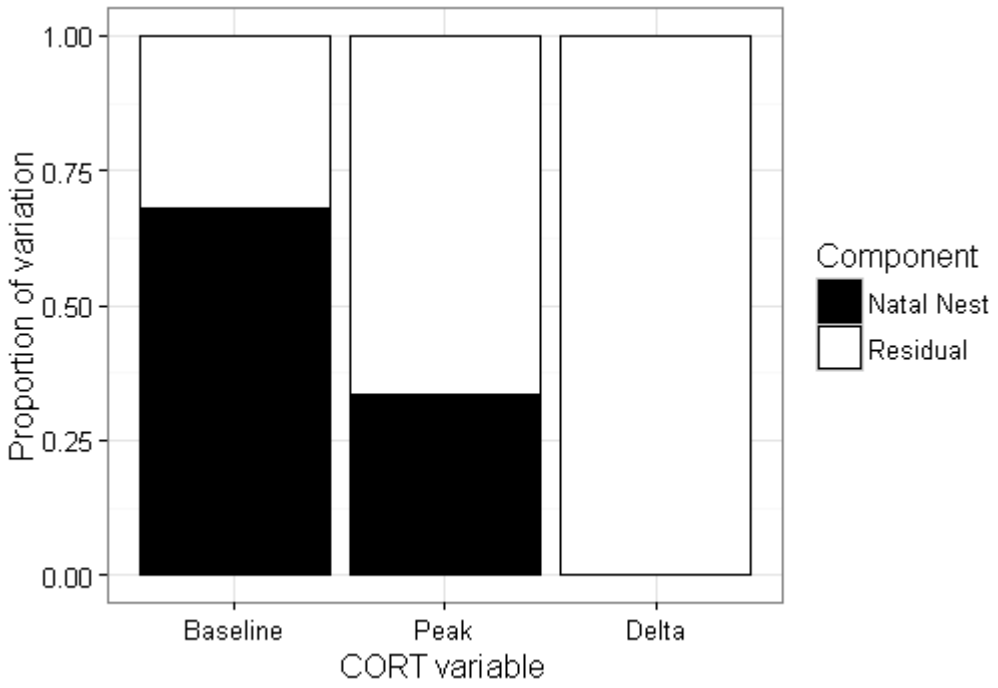

Figure 3.1: The familial and residual components of variation for each of the CORT measures in the starling cohort in experiment 1.

Table 3.1: Output from linear mixed effect models predicting body condition from sex and experimental treatments in experiment 1. A * represents an interaction term.

Model Number	AICc	Dependent Variable	Fixed Predictors	LRT	*P*	n
1	184.1	Body Condition	**Sex**	10.00	**< 0.01***	31
			Amount*Effort	6.18	**0.01***	

3.3.1b Familial Effects

To examine the effects of natal nest (including genetic, very early-life environmental or parental quality effects) on each CORT variable, a variance components analysis was used. A model was fitted with an intercept and a random effect for natal nest for each CORT variable using restricted ML estimation. Natal nest accounted for 68% of the variation in baseline CORT, 33% of the variation in peak CORT and 0% of the variation in ΔCORT (Figure 3.1).

3.3.1c Developmental Treatment

We ran a series of mixed effects models predicting each CORT variable (Baseline, Peak, ΔCORT) from the experimental treatments (Amount, Effort) as both additive and interacting factors (Table 3.2). There were no significant effects of either treatment on baseline CORT (Table 3.2, Models 2-3; Figure 3.2a). There was a significant interaction of Amount by Effort on peak CORT concentrations (Table 3.2, Models 4-5), with birds from the Plenty-Hard treatment groups producing significantly less peak CORT than other birds (Figure 3.2b). There was also a significant effect of body condition on peak CORT, with birds in better condition producing more peak CORT in response to the stressor. There was a marginally significant effect of baseline CORT concentrations on peak CORT. Birds with lower levels of baseline CORT were more likely to also produce lower concentrations of peak CORT. There was an effect of Effort, but not Amount, on ΔCORT (Table 3.2, Models 6-7). Birds from Hard treatment groups had more positive values of ΔCORT, showing a faster return of CORT to baseline conditions by Time 30 (Figure 3.2c). Despite Figure 3.2c appearing to show an important effect, Amount did not have a significant effect on ΔCORT when the model included CORT at Time 15 as a covariate. When removing this covariate, Amount had a marginally significant effect on ΔCORT (Amount LRT = 2.66, *P* = 0.10~). Finally, birds with increased CORT at Time 15 had a significantly higher value of ΔCORT overall.

Table 3.2: Output from linear mixed effect models predicting CORT variables from experimental treatments in experiment 1. A * represents an interaction term.

Model Number	AICc	CORT Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
2	129.5	Baseline	Amount*Effort	0.01	0.92	0.08 (0.84)	31
3	126.4	Baseline	Amount	< 0.01	> 0.99	< 0.01 (0.42)	31
			Effort	0.20	0.65	0.19 (0.42)	
4	228.4	Peak	Cort0	3.52	0.06~	1.37 (0.70)	31
			Amount*Effort	4.91	**0.03***	-10.01 (4.33)	
			Body Condition	4.16	**0.04***	0.70 (0.30)	
			Sex	0.30	0.58	1.83 (3.12)	
5	224.7	Peak	Cort0	3.56	0.06~	1.35 (0.69)	31
			Amount*Effort	4.82	**0.03***	-10.06 (4.40)	
			Body Condition	7.36	**< 0.01***	0.79 (0.27)	
6	197.1	Delta	**Cort15**	25.84	**< 0.01***	0.69 (0.09)	31

			Amount*Effort	1.60	0.21	3.47 (2.65)	
7	195.3	Delta	**Cort15**	24.35	**< 0.01***	0.66 (0.10)	31
			Amount	0.20	0.66	-0.63 (1.42)	
			Effort	4.11	**0.04***	2.91 (1.36)	

3.3.1d Telomere Attrition

Experimental treatments were replaced with DTA (Table 3.3). There was no effect of DTA on any CORT variable (Table 3.3, Models 8-11; Figure 3.2d-f).

Table 3.3: Output from linear mixed effect models predicting CORT variables from DTA in experiment 1.

Model Number	AICc	CORT Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
8	103.0	Baseline	DTA	0.23	0.63	-0.48 (0.98)	26
9	185.2	Peak	Cort0	0.133	0.72	0.27 (0.72)	26
			Body Condition	5.01	**0.03***	1.01 (0.35)	
			Sex	0.19	0.67	1.57 (2.77)	
			DTA	0.82	0.37	-5.01 (5.13)	
10	181.7	Peak	Cort0	0.11	0.74	0.23 (0.68)	26
			Body Condition	12.81	**< 0.01***	1.18 (0.28)	
			DTA	1.28	0.26	-6.17 (5.18)	
11	167.3	Delta	**Cort15**	15.75	**< 0.01***	0.57 (0.12)	26
			DTA	0.15	0.70	-1.45 (3.72)	

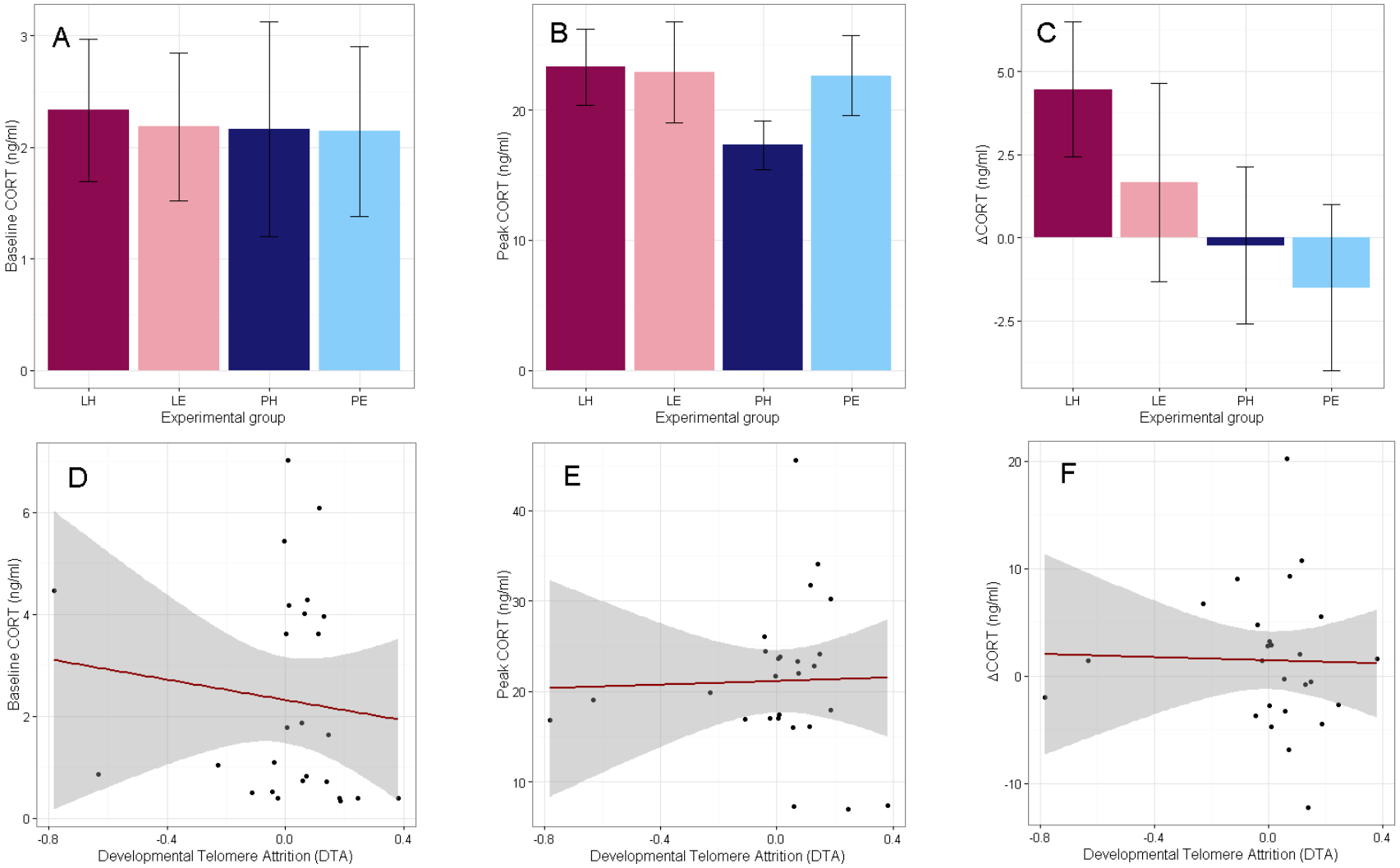

Figure 3.2: A) B) C) – The mean effect of developmental treatments on baseline CORT (A), peak CORT (B) and ΔCORT (C) in experiment 1. Experimental treatment groups were LH (Lean-Hard), LE (Lean-Easy), PH (Plenty-Hard) and PE (Plenty-Easy). Error bars represent one standard error of the mean. D) E) F) – The effect of developmental telomere attrition (DTA) on baseline CORT (D), peak CORT (E) and ΔCORT (F) in experiment 1. More negative DTA indicates greater attrition. ΔCORT is the change in CORT between Time 15 and Time 30 where a positive value indicates a reduction in CORT.

3.3.2 Experiment 2

The stress response measurements presented in this section refer to the second stress series conducted when the birds were 18 months of age.

3.3.2a Sex and Body Condition

Mass and body condition were again significantly correlated in experiment 2 (r = 0.97, *P* < **0.01***). Males had a significantly, albeit modestly, higher body condition than females (mean males 1.0, mean females -3.75, Table 3.4) but there was no effect of developmental treatments on body condition (mean LE 2.05 mean LH -2.46, mean PE -1.55, mean PH -3.31, Table 3.4).

Table 3.4: Output from linear mixed effect models predicting body condition from sex and experimental treatments in experiment 2. A * represents an interaction term.

Model Number	AICc	Dependent Variable	Fixed Predictors	LRT	*P*	n
12	189.3	Body Condition	Sex	3.59	0.06~	30
			Amount*Effort	0.76	0.38	
13	186.6	Body Condition	**Sex**	3.91	**0.05***	30
			Amount	0.17	0.68	
			Effort	0.80	0.37	

3.3.2b Familial Effects

Natal nest accounted for 2% of the variation in baseline CORT, 21% of the variation in peak CORT and 40% of the variation in ΔCORT (Figure 3.3).

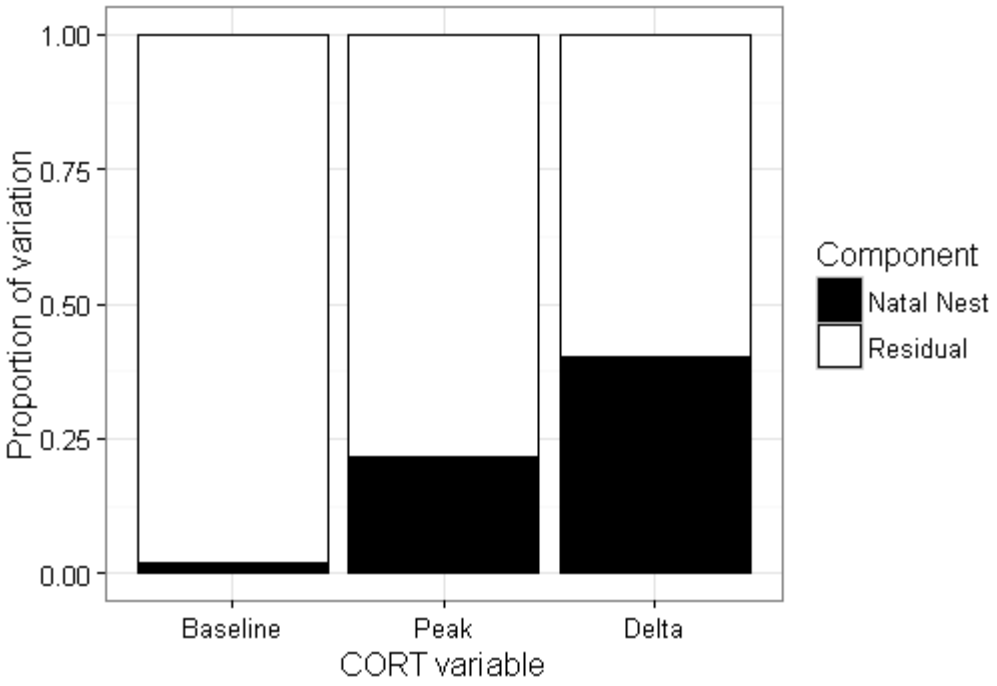

Figure 3.3: The familial and residual components of variation for each of the CORT measures in the starling cohort in experiment 2.

3.3.2c Developmental Treatment

Developmental treatments were used in mixed effects models as predictors for each CORT variable in experiment 2 (Table 3.5). Neither Amount nor Effort predicted baseline CORT or peak CORT levels, nor was there an interaction between the treatments (Table 3.5, Model 13-16; Figure 3.4a-b). Baseline CORT did not have an effect on Peak CORT levels. There was a significant interaction between the Amount and Effort treatments on ΔCORT (Table 3.5, Model 17). Birds from the Plenty condition differed in the direction of ΔCORT, with Plenty-Easy birds showing more positive ΔCORT (representing a reduction in CORT from 15 minutes to 30 minutes) and Plenty-Hard birds showing more negative ΔCORT (a further increase in CORT from 15 minutes to 30 minutes) (Figure 3.4c). Finally, there was an effect of CORT at Time 15 on ΔCORT. Birds with higher CORT at Time 15 were more likely to show positive ΔCORT values.

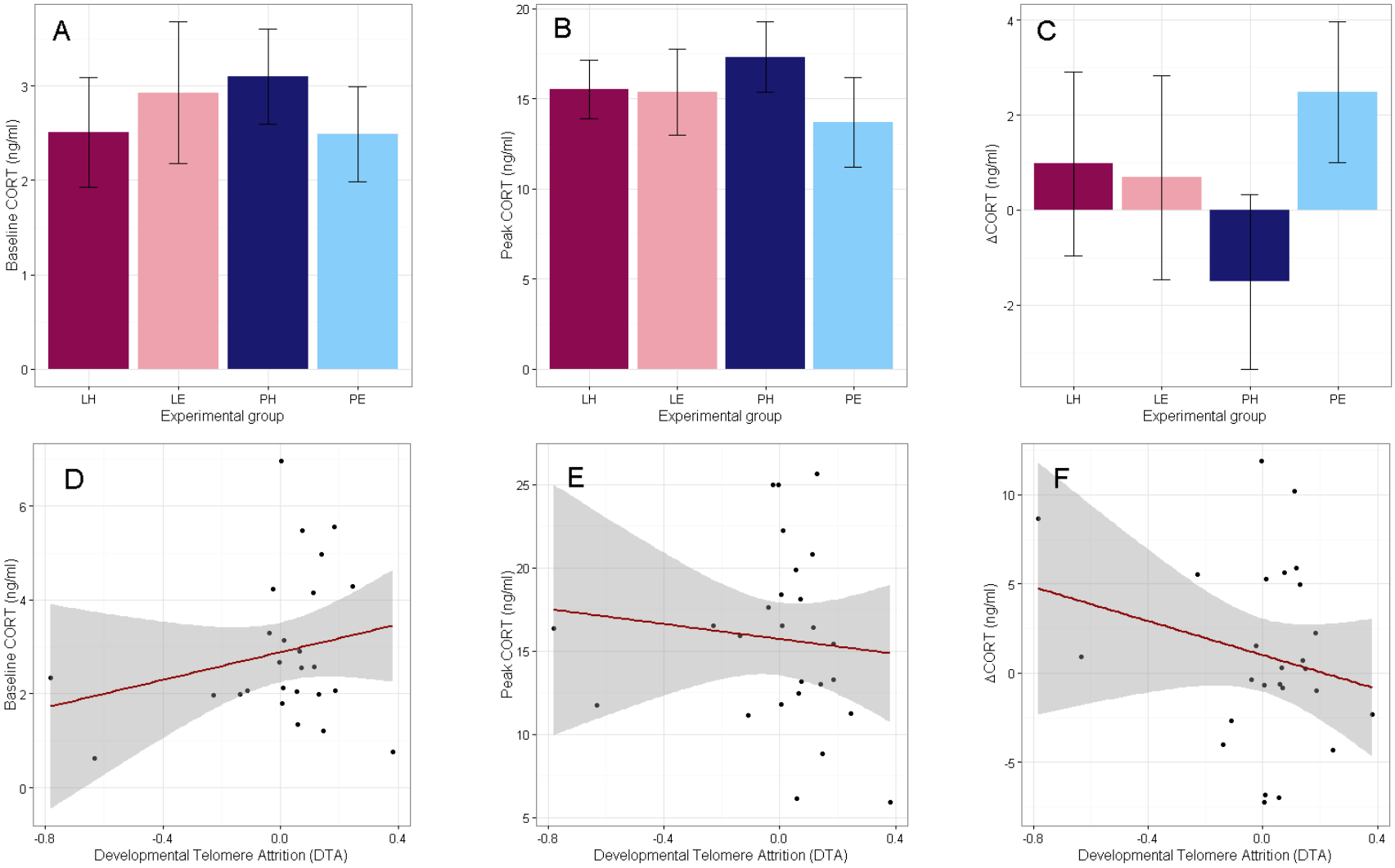

Figure 3.4: A) B) C) – The mean effect of developmental treatment on baseline CORT (A), peak CORT (B) and ΔCORT (C) in experiment 2. Experimental treatment groups are LH (Lean-Hard), LE (Lean-Easy), PH (Plenty-Hard) and PE (Plenty-Easy). Error bars represent one standard error of the mean. D) E) F) – The effect of developmental telomere attrition (DTA) on baseline CORT (D), peak CORT (E) and ΔCORT (F) in experiment 2. More negative DTA indicates greater attrition. ΔCORT is the change in CORT between Time 15 and Time 30 where a positive value indicates a reduction in CORT.

Table 3.5: Output from linear mixed effect models predicting CORT variables from experimental treatments in experiment 2. A * represents an interaction term.

Model Number	AICc	CORT Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
14	124.5	Baseline	Amount*Effort	0.88	0.35	1.03 (1.09)	30
15	122.2	Baseline	Amount	0.02	0.89	0.08 (0.55)	30
			Effort	0.06	0.81	0.13 (0.55)	
16	203.2	Peak	Cort0	1.85	0.17	0.95 (0.64)	30
			Amount*Effort	0.45	0.50	2.51 (3.70)	
17	200.2	Peak	Cort0	2.31	0.13	1.06 (0.63)	30
			Amount	< 0.01	0.99	<-0.01 (1.85)	
			Effort	1.00	0.32	1.87 (1.84)	
18	176.5	Delta	**Cort15**	14.35	**< 0.01***	0.42 (0.10)	30
			Amount*Effort	4.08	**0.04***	-4.36 (2.08)	

3.3.2d Telomere Attrition

Experimental treatments were replaced with DTA. There was no effect of developmental telomere attrition on any CORT variable (Table 3.6, Models 18-20; Figure 3.4d-f).

Table 3.6: Output from linear mixed effect models predicting CORT variables from DTA in experiment 2.

Model Number	AICc	CORT Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
19	104.7	Baseline	DTA	1.55	0.21	1.58 (1.22)	26
20	168.5	Peak	Cort0	0.99	0.32	0.70 (0.62)	26
			DTA	0.22	0.64	-1.99 (3.98)	
21	150.5	Delta	**Cort15**	15.6	**< 0.01***	0.56 (0.12)	26
			DTA	0.11	0.74	-0.90 (2.62)	

3.3.3 Repeatability and Effects of Time

3.3.3a Intra-Class Correlations

Repeatability between baseline CORT, peak CORT and ΔCORT measures from experiment 1 and experiment 2 were calculated using intra-class correlations (Table 3.7). Across all birds, baseline CORT and peak CORT measures were found not to be repeatable within-individuals (Table 3.7, Correlation 1-2). ΔCORT, however, had a significant, but modest, degree of repeatability (Table 3.7, Correlation 3). We then looked at differences in repeatability within the experimental treatment groups. There was no repeatability between baseline CORT and peak CORT from the experiments in any treatment group (Table 3.7). ΔCORT was repeatable in Plenty and Hard groups (Table 3.7, Correlation 6 and Correlation 15), but not in Lean or Easy groups.

Table 3.7: Output from intra-class correlations (ICC) assessing repeatability between baseline CORT, peak CORT and ΔCORT measures from experiment 1 and experiment 2. Repeatability was assessed for each treatment group (Plenty, Lean, Easy and Hard) and for all birds (Overall). NB: small negative values for the ICC estimator can occur under some circumstance and are taken to indicate that the true repeatability is approximately zero.

Correlation Number	Group	CORT Variable	ICC Value	F Value	*P*	n
1	Overall	Baseline	0.13	1.29	0.25	30
2	Overall	Peak	-0.03	0.94	0.56	30
3	Overall	Delta	0.32	1.96	**0.04***	30
4	Plenty	Baseline	0.07	1.14	0.40	16
5	Plenty	Peak	< 0.01	0.99	0.51	16
6	Plenty	Delta	0.56	3.58	**< 0.01***	16
7	Lean	Baseline	0.24	1.64	0.19	14
8	Lean	Peak	-0.02	0.96	0.53	14
9	Lean	Delta	0.07	1.15	0.40	14
10	Easy	Baseline	0.30	1.84	0.13	15
11	Easy	Peak	-0.06	0.89	0.58	15
12	Easy	Delta	0.23	1.59	0.19	15
13	Hard	Baseline	-0.02	0.96	0.53	15
14	Hard	Peak	0.13	1.30	0.32	15
15	Hard	Delta	0.53	3.23	**0.02***	15

3.3.3b Time of Stress Response Measurement

The effect of time on each CORT variable was assessed by combining data from experiment 1 and experiment 2 and fitting models with developmental treatments (Amount, Effort) and experiment (1 or 2) as predictors (Table 3.8). Time to sample had a significant positive effect on baseline CORT, however there were no overall effects of developmental treatments nor experiment (Table 3.8, Models 21-23). There was a significant individual effect of experiment on peak CORT. In experiment 2, birds had significantly lower levels of peak CORT when compared to experiment 1 (Table 3.8, Model 26, mean experiment 1: 20.88, mean experiment 2: 15.46). Baseline CORT also predicted peak CORT levels with birds with higher baseline CORT again showing higher peak CORT overall (Table 3.8, Model 24-26). There was a three-way interaction between Amount, Effort and experiment on ΔCORT (Table 3.8, Model 27). This was caused by the Amount by Effort interaction seen in experiment 2 but not in experiment 1 (Figure 3.2c, Figure 3.4c). Finally, CORT at Time 15 predicted ΔCORT, with higher CORT at Time 15 leading to faster rates of recovery (Table 3.8, Model 27).

Table 3.8: Output from linear mixed effect models predicting CORT variables from experimental treatments and experiment. Where abbreviated, A = Amount, E = Effort. A * represents an interaction term.

Model Number	AICc	CORT Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
22	253.6	Baseline	**Time to Sample**	7.25	**< 0.01***	0.02 (<0.01)	30
			A*E*Experiment	0.07	0.79	0.37 (1.39)	
23	250.5	Baseline	**Time To Sample**	7.64	**< 0.01***	0.02 (<0.01)	30
			Amount*Effort	0.52	0.47	0.50 (0.69)	
			Amount*Experiment	< 0.01	0.96	-0.03 (0.70)	
			Effort*Experiment	0.14	0.71	0.26 (0.69)	
24	242.6	Baseline	**Time To Sample**	7.40	**< 0.01***	0.02 (<0.01)	30
			Amount	0.18	0.67	0.15 (0.35)	
			Effort	0.12	0.73	0.12 (0.35)	
			Experiment	0.10	0.75	0.12 (0.37)	
25	430.5	Peak	**Cort0**	3.89	**0.05***	1.02 (0.50)	30
			Body Condition	2.41	0.12	0.29 (0.19)	
			A*E*Experiment	1.91	0.17	8.53 (6.13)	

26	428.0	Peak	**Cort0**	4.00	**0.05***	1.06 (0.51)	30
			Amount*Effort	0.27	0.60	-1.64 (3.14)	
			Amount*Experiment	0.73	0.39	2.67 (3.13)	
			Effort*Experiment	1.90	0.17	4.36 (3.13)	
27	422.2	Peak	**Cort0**	3.93	**0.05***	1.07 (2.14)	30
			Amount	0.80	0.37	-1.44 (1.60)	
			Effort	0.01	0.90	-0.19 (1.60)	
			Experiment	14.91	**< 0.01***	-6.72 (1.62)	
28	369.7	Delta	**Cort15**	42.18	**< 0.01***	0.60 (0.07)	30
			A*E*Experiment	4.01	**0.05***	-7.43 (3.55)	

Developmental treatment was then replaced with DTA (Table 3.9). There was no effect of DTA, nor experiment on overall baseline CORT (Table 3.9, Models 28-29). There was a marginally significant effect of experiment on peak CORT (Table 3.9, Model 31) and a highly significant effect of experiment on ΔCORT (Table 3.9, Model 33).

Table 3.9: Output from linear mixed effect models predicting CORT variables from DTA and experiment. A * represents an interaction term.

Model Number	**AICc**	**CORT Variable**	**Fixed Predictors**	**LRT**	***P***	**B (± SE)**	**N**
29	211.0	Baseline	**Time to Sample**	4.07	**0.04***	0.02 (<0.01)	27
			DTA*Experiment	2.10	0.15	2.30 (1.57)	
30	210.2	Baseline	**Time To Sample**	4.23	**0.04***	0.01 (<0.01)	27
			DTA	0.44	0.51	0.60 (0.90)	
			Experiment	0.29	0.59	0.22 (0.41)	
31	357.4	Peak	Cort0	0.71	0.40	0.48 (0.53)	27
			Body Condition	3.97	**0.05***	0.39 (0.19)	
			DTA*Experiment	0.22	0.64	-3.19 (6.71)	
32	354.7	Peak	Cort0	0.58	0.45	0.42 (0.52)	27
			Body Condition	4.14	**0.04***	0.40 (0.19)15	

			DTA	0.06	0.80	-0.96 (3.72)	
			Experiment	3.22	0.07~	-3.52 (1.93)	
33	312.5	Delta	**Cort15**	33.11	**< 0.01***	0.61 (0.08)	27
			DTA*Experiment	0.28	0.60	-2.27 (4.31)	
34	310.0	Delta	**Cort15**	33.34	**< 0.01***	0.61 (0.08)	27
			DTA	0.22	0.64	-1.12 (2.40)	
			Experiment	5.25	**0.02***	2.69 (1.12)	

3.4 Discussion

3.4.1 Summary of Results

The aim of this study was to determine how differential experience of two naturally relevant forms of early-life adversity affect functioning of the adult HPA axis in European starlings. We measured stress responsiveness at two time points, in which birds differed in age by approximately 14 months. We predicted that birds exposed to more adversity as juveniles would show a greater response to an acute stressor in both experiments. Our results do not support this in experiment 1, however there is some evidence that meets this prediction in experiment 2. In experiment 1, we found a significant interaction of Amount by Effort on peak CORT concentrations, with birds from Plenty-Hard treatment groups producing lower levels of peak CORT. We also found an effect of Effort on ΔCORT, with stronger negative feedback and a faster return to baseline CORT levels in birds from Hard treatment groups. In experiment 2, treatment effects on peak CORT had disappeared, however an Amount by Effort interaction was seen on ΔCORT. Here, Plenty-Easy birds now showed stronger negative feedback whereas CORT levels continued to rise in Plenty-Hard birds. Throughout both experiments, there were no effects of telomere attrition on any CORT variable. Finally we compared data from the two experiments and predicted that the stress response would show some degree of repeatability within individuals, however this was only true for ΔCORT. There was an effect of experimental timing on peak CORT concentrations, with overall peak CORT significantly decreasing between experiment 1 and experiment 2. We also found a significant Amount by Effort by experiment interaction on ΔCORT, driven by the strong treatment interaction seen in experiment 2 but not in experiment 1.

3.4.2 Developmental Treatment and Telomere Attrition

We found contrasting results between experiment 1 and experiment 2. At 4 months of age, birds from Hard treatment groups had an attenuated stress response, with the strongest effects seen in Plenty-Hard birds. Plenty-Hard birds had lower levels of peak CORT and Hard birds in general had higher levels of ΔCORT (potentially representative of increased negative feedback, reduced adrenal gland secretion, or a faster peak in the CORT cycle) leading to faster cessation of the stress response. These results did not meet our predictions, however evidence from the literature shows that adrenocortical responses in bird species are not uniform. Early adversity has been demonstrated, contrary to our predictions, to have no effect upon or even dampen the adult stress response (Andrews et al in prep; Schwabl 1999; Zimmer et al. 2013; Zulkifi et al. 1995). Kitaysky et al. (2005), for example, showed that puffin chicks experiencing food deprivation for 3 weeks post-hatch had lower levels of peak CORT in response to a stressor when compared to controls.

There are two main theories that could explain this. First, there are environmental situations in which it may be adaptive to suppress HPA activity (Kitaysky et al. 2003). CORT has been shown to elicit increased begging behaviour, which functions to raise parental feed rate and the amount of food delivered (Kitaysky et al. 2001). However in situations where this is not possible, the costs of prolonged CORT exposure (e.g. reduced growth rate, impaired cognitive function) outweigh these benefits. Therefore if a chick is unable to alter parental behaviour through CORT increase, such as in our developmental treatments, selection may favour a reduced stress response. HPA suppression could explain why Hard treatments demonstrate a faster return to baseline CORT levels than Easy birds. It is possible that this adaptation is not easily reversed, and therefore still detectable 4 months after the manipulation ended. Peak CORT concentrations, however, were found to be decreased in only the Plenty-Hard treatment (and not also in Lean-Hard individuals as predicted). Lean-Hard nestlings, receiving the smallest amount of food for their begging effort, may depend on CORT-elicited behaviour such as increased sibling aggression and maximum begging to ensure survival. Therefore in extreme developmental cases, suppression of CORT would not be adaptive. It is interesting to note that there was no main effect of Amount on the adult HPA axis, and this treatment was only significant when interacting with Effort, showing that the effect of one type of adversity may depend on the simultaneous presence of others – that is, different adversities are not additive.

Interestingly, the results from experiment 1 cannot be explained by a measure of biological ageing (we saw no effects of DTA on CORT profiles). Evidence exists to support the theory that biologically older individuals have attenuated stress responses to prioritise reproduction over survival as life expectancy decreases (Heidinger et al. 2006). Nettle et al. (2017) previously showed that increased

DTA (thought to be a measure of biological ageing) was associated with more adverse treatment groups in this cohort. We go on to show that these groups also possess a diminished stress response in experiment 1. However, we found no direct evidence of telomere attrition on the stress response to support this. Using birds from two separate manipulations (small brood vs large brood, largest competitor vs smallest competitor), Andrews et al. (in prep) found that European starlings with greater DTA had a smaller stress response in adulthood (reduced peak CORT and increased ΔCORT). Interestingly, they did not find effects of developmental treatment on the stress response. Our study differs in terms of both the developmental treatments used, but also the age that birds were sampled. Both cohorts in Andrews et al. (in prep) were sampled at intermediate ages to our birds in experiment 1 and experiment 2. Furthermore, as telomeres do not appear to lengthen over time, we would expect biologically older (those exposed to more adversity) individuals to show a similar or even further diminished stress response by experiment 2. This was not found to be the case and therefore we cannot conclude that the reduced stress responses seen in Hard or Plenty-Hard treatment groups in experiment 1 are a result of biological ageing.

By experiment 2, treatment effects on peak CORT were no longer detectable (similar to Pravosudov & Kitaysky 2006). Crino et al. (2014) showed that exposure to CORT during development had an effect on zebra finches at 30 days old (increasing peak CORT concentrations but having no effect on negative feedback), however these treatment differences were no longer measureable at 60 or 90 days post-hatch. Our results echo this (albeit on a different timescale – our first measurement was taken at approximately 130 days), indicating that our treatments may not have long-lived effects on concentrations of peak CORT. We still, however, detected a treatment effect on ΔCORT with data suggesting that Plenty-Easy birds showed stronger negative feedback than Plenty-Hard birds (although no differences were seen between Lean-Easy and Lean-Hard birds). CORT in Plenty-Hard birds continued to increase between Time 15 and Time 30. This experiment partially met our prediction that more adverse treatments would show reduced capabilities of negative feedback, and has also been replicated in the literature (Spencer et al. 2009; Banerjee et al. 2012).

The most efficient stress response in experiment 2 was seen in Plenty-Easy birds, who were exposed to the least adverse environment, as predicted. Similar to this, Banerjee et al. (2012) found that zebra finches fed an *ad lib* diet demonstrated attenuated stress responses. Interestingly, these finches also showed higher expression of GR and mineralocorticoid receptor (MR) mRNA. An explanation for the increased efficiency of the stress response in the Plenty-Easy birds could be that they possess more GR and MR transcripts than other treatment groups, leading to a faster cessation of the stress response. Contrary to our predictions, the least efficient response was not seen in birds that had experienced the most early-life adversity (the Lean-Hard group). Instead, it was the Plenty-Hard birds

that showed prolonged CORT release and reduced negative feedback compared to the other treatment groups. These data suggest that a combination of increased begging effort and the consumption of an unrestricted diet as a juvenile is a risk factor for a hyperactive stress response in adulthood, however the mechanisms that mediate this are still unclear. Interestingly, we did not find any difference in ΔCORT between Lean-Easy and Lean-Hard birds. Begging effort was not shown to have an effect on negative feedback in birds that had received a restricted juvenile diet, but did affect negative feedback in birds that experienced *ad lib* food.

Between experiment 1 and experiment 2, there were two interesting changes regarding developmental treatment effects on ΔCORT. First, there was a switch from a main effect of Effort in experiment 1, to an interaction of Amount by Effort in experiment 2. It is possible that the two sources of adversity affect negative feedback by different mechanisms, which work on different timescales. Second, Hard birds had a more efficient feedback system in experiment 1, as indicated by higher ΔCORT, however this was reversed for one of the Hard treatment groups in experiment 2. Plenty-Hard birds demonstrated a prolonged stress response by experiment 2. Examples of environmentally-mediated reversals have been seen in the literature (Smythe et al. 1996; Maccari et al. 1995). Pre-natal exposure to stress in rats has been shown to impair negative feedback, however Morley-Fletcher et al. (2003) demonstrated that by providing environmental enrichment, they were able to reverse this effect and overall reduce HPA axis reactivity. Weaver et al. (2006) suggest that such reversals may be due to disruption of GR expression, mediated by epigenetic mechanisms. Using methionine (a dietary supplement previously shown to increase DNA methylation (Tremolizzo et al. 2002)), Weaver et al. (2005) were able to reverse the protective effects of high quality maternal care, and could experimentally induce increased acute stress responsiveness in rats. It is possible that different combinations of early-life and adult environments could trigger epigenetic reprogramming of the stress response, however further research into this area is needed.

3.4.3 Repeatability and Ageing

In these birds, we found no repeatability between baseline CORT and peak CORT measures, but modest repeatability for ΔCORT. This is the first study, to our knowledge, that uses a cohort of birds in which all prior experience has been controlled for to test CORT repeatability. It is surprising that our baseline and peak CORT measures were therefore not repeatable within individuals (contrary to Cockrem & Silverin 2002; Kralj-Fišer et al. 2007; Wada et al. 2008). Lendvai et al. (2015) speculate that individual plasticity and how an animal adapts to its surroundings contributes to a difference in CORT profiles within individuals. With respect to ΔCORT, we showed that Easy birds and Lean birds did not demonstrate repeatability, however Plenty birds and Hard birds did. Our data suggests that early-life

experience can moderate the level of plasticity a bird is capable of in adulthood, and specifically how repeatable this makes aspects of the stress response. Significant repeatability in ΔCORT demonstrates that birds showed more similar negative feedback patterns than baseline CORT and peak CORT within-individuals. This is supported in the literature, with studies finding that it is often the negative feedback component that is developmentally programmed with exposure to early-life adversity (Spencer et al. 2009; Kapoor et al. 2006). An alternative interpretation is that baseline and peak CORT measures are not repeatable in the starling specifically, however further studies in this species are needed to conclude this.

Our data show that peak CORT and ΔCORT (but not baseline CORT) are affected by time. This is consistent with literature from a range of species that explain these differences by increasing chronological age (Wilcoxen et al. 2011; Heidinger et al. 2006). Lendvai et al. (2015) show a significant decrease in CORT measured 30 minutes after exposure to a stressor (but no differences in baseline CORT) over a single year in a population of house sparrows (*Passer domesticus*). However, a lack of further CORT measurements do not accurately determine whether peak CORT is decreased or ΔCORT is in fact increased. We show that both components are affected by time. It is possible that our birds have habituated to the standard capture procedure used between the two experimental time points, which would lead to a smaller stress response. Support for this comes from Lynn et al. (2010), who found that Eastern bluebirds (*Sialis sialis)* have reduced CORT responses after a single capture event. However, Lendvai et al. (2015) stress that naïve birds in their experiment did not have higher stress response measurements when compared to pre-exposed age-matched individuals, as would be predicted. Habituation to the laboratory environment and the individual cages used in experiments could also reduce the size of an individual's stress response. Lendvai et al. (2007) demonstrated that individuals are capable of modulating their stress response in response to their environment, for example, by suppression during breeding. A second theory for why peak CORT is shown to decrease over time is that the ability to produce a strong stress response declines with age. Andrews et al. (in prep) investigated this theory, and show that birds with greater biological age in fact strengthen their negative feedback capacity, providing evidence against a senescence hypothesis.

3.4.4 Conclusions and the Bigger Picture

This European starling cohort and our developmental manipulation has allowed us to study specific components of early-life adversity and their effects on the adult stress response, with the opportunity to take repeated physiological measures at different ages. Methodologically, we have identified issues with the repeatability of CORT measures and this should be taken into account when interpreting stress physiology data. We have shown that our treatments can lead to long-lasting alterations to the

HPA axis, but these effects are not necessarily stable and are shown to change over time. Our results indicate that certain amounts of early-life adversity result in a change to the HPA axis, measurable at 4 months of age, but this wears off with time as animals adjust to their adult environment. We predicted that early-life adversity would result in a hyperactive stress response, however the results shown in this study demonstrate a more complicated picture. Throughout, we found that both Amount and Effort contributed to HPA modification, as both additive and interactive predictors. Being able to tease apart different sources of adversity has shown that different combinations of environmental experience can have specific effects on components of the stress response.

Of particular interest was the finding that changes to ΔCORT were long-lasting (detectable at 18 months after the developmental manipulation ended), with Plenty-Hard birds showing prolonged release of CORT. Remarkably, problems with the negative feedback system of the stress response is present in approximately half of patients suffering from depression (Weiler et al. 1982; Carroll et al. 1981). Our study raises the question of whether Plenty-Hard birds also show depressive or anxious phenotypes, which would contribute to evidence that early-life adversity can lead to the development of mood disorders through alteration to the HPA axis. We can continue to investigate mood, emotion and stress physiology throughout the lives of these birds, taking into account further physiological and behavioural measures to provide a comprehensive picture of the effects of early-life adversity on the adult phenotype.

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Chapter 4. Stressor Reactivity and Anxiety-Like Behaviour

Abstract

Early-life adversity has been linked to the development of mood disorders such as anxiety, however it is not possible to test this experimentally in humans. Inducing anxiety in animals is possible through the use of auditory stimuli that signal a threat. Anxiety is often measured with respect to feeding behaviour. However, early-life adversity has previously been shown to alter hunger and motivation to feed, making interpretation of food-related behaviour as a measure of anxiety difficult. It is also unknown how different sources of adversity can affect anxiety-like behaviour in adulthood. In a cohort of hand-reared European starlings (*Sturnus vulgaris*), a two-by-two factorial design was used to simulate different levels of early-life adversity by separately manipulating food availability (Amount – Plenty vs Lean) and begging effort (Effort – Easy vs Hard) in the first two weeks post-hatch. We measured a suite of behavioural responses of the adult birds when presented with and without an acute stressor (a conspecific distress call). Anxiety-like behaviour was measured in the context of the introduction of a deprived resource in two experiments. The first used a bowl of food and the second used a water bath. We found that the distress call successfully induced anxiety. Adverse developmental treatments were shown to increase anxiety measured by behaviours such as latency to move (experiment 1) and vigilance whilst feeding (experiment 1 and 2). We found that birds with greater developmental telomere attrition (a biomarker of biological age) also showed increased anxiety in experiment 2, demonstrated by increased latencies to move and less time spent bathing. Finally, we also showed associations between physiological measures (baseline corticosterone and the change in CORT between 15 and 30 minutes - ΔCORT) and anxiety. Both an increase in baseline CORT and ΔCORT was associated with anxiogenic behaviour. We conclude that both nutritional restriction and increased begging effort can increase anxiety-like behaviour in adult starlings, and this could be mediated by changes to the HPA axis.

4.1 Introduction

Early-life adversity is a well-known predictor of altered behavioural and physiological phenotypes and has been repeatedly linked to the development of affective disorders in humans such as anxiety (including panic disorder, obsessive-compulsive disorder and generalised anxiety disorder) (Portegijs et al. 1996; Stein et al. 1996; Heim & Nemeroff 2001; Heim & Nemeroff 1999). Anxiety is an essential emotion necessary for survival and can be defined as "a physiological and behavioural state induced by an actual or perceived threat to well-being" (Steimer 2002). However, issues can occur when anxiety is excessive, persistent or present in inappropriate situations (Ohl et al. 2008; Staples 2010). The development of anxiety has been linked to both genetic predispositions and to the influence of environmental factors such as chronic stress exposure, particularly in early life. For example, children who have suffered from emotional maltreatment are often diagnosed with anxiety disorders in adulthood (Stein et al. 1996; Kuo et al. 2011). However, the studies by Stein et al. and Kuo et al. are purely correlational. Animal models provide a way to experimentally test the hypothesis that early-life adversity can leave individuals susceptible to the development of anxiety disorders.

Distinct behavioural and physiological patterns exist in animals that indicate the presence of anxiety, for example increased freezing behaviour, restlessness, corticosterone release and vigilance (Lang et al. 2000). When measuring anxiety in animals, the distinction between baseline anxiety and anxiety exhibited under threat is important. Baseline anxiety is shown in low-threat situations and is an underlying and enduring characteristic of an animal. In high-threat situations, a different type of anxiety is demonstrated in response to an anxiogenic stimulus (Beuzen & Belzung 1995). Several authors have demonstrated that underlying baseline anxiety is increased in adult rats that have experienced maternal separation, characterised by greater latencies to explore and to approach and eat food (Kalinichev et al. 2002; Caldji et al. 2000). Testing anxiety exhibited under threat can be done by evoking defensive behaviour and risk assessment strategies (Blanchard et al. 1993). Commonly, this has been tested in rodents through exposure to a potential predator by either physical presence, an odour or a predatory call (Eilam et al. 1999; Adamec & Shallow 1993). However, the study of both baseline and threat-induced anxiety is limited in birds.

Similar to mammals, Brilot et al. (2009a) showed that auditory stimuli (here, starling alarm calls – produced when a predator has been detected) are sufficient to generate an anxiety-like state in birds, evidenced by an increase in freezing behaviour and a reduction in time spent using a food bowl. An alternate auditory stimulus known to affect behaviour in conspecifics is a distress call. In birds, distress calls are produced when birds are under severe threat, for example whilst being captured by a predator (Frings & Jumber 1954). In response to this call, starlings have been shown to display more

anxiety-like behaviour (measured by an increased latency to return to a feeding site) (Conover & Perito 1981). The effect of early-life adversity with respect to anxiety-like behaviour produced in response to an auditory stimulus, however, has not been studied in birds.

Early-life stress exposure has been suggested to leave individuals susceptible to anxiety disorders through alterations to the hypothalamic-pituitary-adrenal (HPA) axis and circulating glucocorticoid concentrations (Vreeburg et al. 2010; Kallen et al. 2008; Condren et al. 2002; Erhardt et al. 2006). Increased glucocorticoid hormones (corticosterone (CORT) found in birds, reptiles and amphibians and cortisol in most mammals and fish) are thought to promote anxiety-like behaviour (Fan et al. 2014; Mitra & Sapolsky 2008). Individual differences in CORT concentrations and HPA reactivity may determine how an animal responds to a stressor, and ultimately influence pathological anxiety development, and therefore should be considered in studies of anxiety. Previously in this cohort, we have shown that the HPA axis can also be influenced by early-life adversity with long-lasting changes seen to a measure reflective of the negative feedback system (ΔCORT) (Chapter 3, Experiment 2). Tying in this physiological data with a behavioural study of anxiety would add to the literature concerning early-life adversity, the development of anxiety disorders and the potential mechanisms that mediate this.

Often, anxiety is measured with respect to feeding behaviour, with latencies to approach a food resource commonly tested. Bloxham et al. (2014) demonstrated that early-life adversity can affect feeding motivation in animals, with birds from high-competition broods more likely to consume toxic prey. This makes interpretation of latency to approach food as a measure of anxiety difficult, as differences in food motivation may confound displays of anxiety-like behaviour. When examining the effects of the early environment, tests for anxiety that include non-food related behaviour are crucial for making robust conclusions. An alternate desirable resource for starlings is access to a water bath. Bathing is an essential behaviour that enables feather maintenance, known to affect escape flight performance (Swaddle et al. 1996). Brilot et al. (2009b) exposed starlings to a loud acoustic stimulus and measured escape flight. They showed that birds with recent access to water baths displayed different escape flight patterns when tested in an aerial maze by increasing flight accuracy but decreasing overall speed. The authors hypothesised that bathing could reduce the perceived risk of the acoustic stimulus through an improvement in flight performance. Brilot & Bateson (2012) went on to demonstrate that birds deprived of bathing water interpreted a conspecific alarm call as a greater risk than birds with access to water baths, evidenced by increased latency to feed and increased vigilance behaviour. It would therefore be interesting to measure the effects of early-life adversity on anxiety with respect to both food and bath deprival in birds.

The aim of our study was to investigate the relationship between early-life adversity and anxiety-like behaviour in adult European starlings (*Sturnus vulgaris*). In a cohort of hand-reared birds, a two-by-two factorial design was used to simulate different levels of early-life adversity by separately manipulating food availability (Amount – Plenty or Lean) and begging effort (Effort – Easy or Hard) (Nettle et al. 2017; Chapter 2). Upon the end of the manipulation, birds were raised in uniform conditions until the time of the adult behavioural experiment. Developmental telomere attrition (DTA), a biomarker of biological age, was measured in erythrocytes and was found to be accelerated in birds who had experienced the lowest amount of food and the highest begging effort (Nettle et al. 2017; Chapter 2). Additionally, CORT profiles were measured 4 months previous to the behavioural experiment, with long-lasting developmental treatment effects seen, particularly on ΔCORT (Chapter 3, Experiment 2).

Here, we report on a suite of behavioural responses of the adult European starlings from the 2014 cohort presented with and without an acute stressor, a conspecific distress call, to measure both baseline anxiety, and anxiety exhibited under threat. We predicted that starlings would show greater anxiety-like behaviour overall when exposed to the call. Anxiety-like behaviour was measured with respect to the introduction of a desirable resource that had previously been deprived. For experiment 1, this was a bowl of *ad libitum* food and for experiment 2, this was access to a water bath. We predicted that birds from more adverse treatment groups and with more DTA would show greater underlying anxiety-like behaviour. Finally, we explored the relationship between adult CORT profiles and anxiety, predicting that more anxious behaviour would be seen in birds with higher baseline CORT, regardless of whether an acoustic stimulus was heard. We predicted that higher peak CORT and lower ΔCORT would be associated with more anxiety-like behaviour when exposed to the distress call.

4.2 Methods

4.2.1 Subjects, Housing and Husbandry

Subjects in this study were adult European starlings that had been subjected to a unique developmental manipulation described elsewhere (Chapter 2; Nettle et al. 2017) designed to dissociate the effects of overall quantity of food received (Amount – Plenty or Lean) from the begging investment required to obtain it (Effort – Easy or Hard) creating 4 experimental groups (Plenty-Easy, Plenty-Hard, Lean-Easy, Lean-Hard). Anxiety-like behaviour was measured when birds were approximately 24 months old.

For the reactivity tests, starlings were transferred to individual experimental cages (75x45x45cm) that served for both reactivity testing and as their home cages for 7 days. Birds were visually, but not

acoustically, isolated. The experimental room was designed to house up to groups of eight birds at a time on two rows of shelves. Cage position (upper and lower) were counter-balanced with respect to developmental treatment. The experiment was conducted in four replicates over a continuous four week time period. The temperature and lighting conditions were kept consistent with those of the indoor home aviaries. Birds were weighed upon entry and exit to the experiment to gain a measure of body mass and body condition (calculation described in chapter 2) at the time of the experiment. Outside of experimental testing periods, all birds had access to two wooden perches, two drinking bottles, a filled water bath and a bowl containing *ad libitum* food (10g cat biscuit, 5g turkey crumb, 5g Orlux, 10 mealworms and 1/8 of a piece of fruit replaced daily).

4.2.2 Experimental Set-Up

Birds were given three days to habituate to the experimental cages. Reactivity was measured over four consecutive testing days in two separate experiments (experiment 1 – 2 days, experiment 2 – 2 days). In each experiment, a testing day comprised of the following: the laboratory lights were switched off without warning at 3pm daily. The experimenter entered the room using only torchlight and introduced a resource to the centre of each cage with as little disturbance to the animals as possible. The experimenter then switched on two video cameras (each trained on four cages) and the test speaker and left the room. The speaker was placed in the centre of the experimental room such that the sound pressure level was an average amplitude of 78.2DB measured at the perch furthest from the speaker (all cages were approximately equidistant from the speaker). Birds were allowed to settle for 3 minutes in the dark.

The independent variable modified in both experiment 1 and experiment 2 was the presence or absence of a distress call. In half of the trials in experiment 1 and experiment 2 (‘Call’ trials), a 10 second distress call was played remotely by the experimenter outside of the room using an Apple iPhone SE and a wirelessly connected Bluetooth speaker (Victorstar, SB510). The same recording was used for all ‘Call’ trials. The distress call was recorded prior to the experiment, and was one of the bird’s response to being handled by the experimenter. In the wild, this call is often produced in response to a predator (Frings & Jumber 1954). The ‘Call’ stimulus was never played on two consecutive testing days, and no bird heard the ‘Call’ stimulus on more than two occasions to avoid habituation to the distress call. For the other half of trials in experiment 1 and experiment 2 (‘No Call’ trials), there was a 10 second period of silence. Presentation of the acoustic stimulus or of silence was counter-balanced with respect to experiment 1 and 2. After this, the lights were immediately switched back on and behaviour was recorded for a minimum of 25 minutes.

Experiment 1 and 2 differed with respect to the introduced resource designed to test anxiety-like behaviour. In experiment 1, the introduced resource consisted of a bowl of *ad libitum* food and in experiment 2, the resource was a water bath filled with cold clean water. Birds had been deprived of the resource before testing. For experiment 1, bowls were removed 6 hours before and for experiment 2, water baths were removed approximately 24 hours before testing. The order of the experiment was fixed; experiment 1 always preceded experiment 2. An example set-up of these combinations are presented in Figure 4.1, with 15 birds receiving combination a), and 15 birds receiving combination b).

a)	Testing Day 1	Testing Day 2	Testing Day 3	Testing Day 4
Resource (Fixed)	'Food'	'Food'	'Bath'	'Bath'
Stimulus (Counterbalanced)	'Call'	'No Call'	'Call'	'No Call'

b)	Testing Day 1	Testing Day 2	Testing Day 3	Testing Day 4
Resource (Fixed)	'Food'	'Food'	'Bath'	'Bath'
Stimulus (Counterbalanced)	'No Call'	'Call'	'No Call'	'Call'

Figure 4.1: The two possible experimental setups in the stress reactivity study. The experiment consisted of 4 testing days. The resource order was fixed, with all birds undergoing experiment 1 on testing days 1 and 2, and experiment 2 on testing days 3 and 4. The stimulus presentation order was counterbalanced. Half of the birds received 'call' trials first (combination a), and the remaining half received 'no call' trials first (combination b).

Birds were not in acoustic isolation during the experiment and were subject to disturbance inside and outside of the experimental room. However, no birds emitted alarm calls in response to either the experimenter or the distress call stimulus. Small disturbances such as husbandry staff walking in the corridor outside of the experimental room were kept to a minimum but could not be completely eliminated. These noises were not novel to the birds as the experimental room lay on the same corridor as the home aviaries.

4.2.3 Behavioural Scoring and Variable Selection

Complete sets of 25 minutes of behavioural data were collected for all 30 birds. Digital files were then scored by a single experimenter in ELAN (The Language Archive, Nijmegen, Sloetjes & Wittenburg 2008). In both experiments, the following behaviours were scored following the lights coming on: latency to move, cage position location and duration (options of perch, wall, paper, bath or bowl),

total number of position changes and duration of time spent using the resource. For experiment 1, the duration of time spent with the bill continuously above or below horizontal when feeding (Head-Up/Head-Down duration) as well as the number of Head-Up and Head-Down transitions were also measured. Variables of interest were identified using information from previous studies in our lab and from a general review of relevant literature and are summarised in Table 4.1. We aimed to capture different aspects of anxiety and therefore chose a suite of behaviours to measure. Briefly, we looked at rate of movement, duration of time spent on the wall, latency to move and average time spent using the resource. In experiment 1, we also looked at two aspects of vigilance behaviour when using the food bowl: head-up proportion (length of time a bird spent with its head above the horizon whilst feeding) and head-change rate (the number of times a bird changed from head-up to head-down behaviour). Head-change rate and head-up proportion were significantly correlated, however this relationship was not strong ($r = 0.38$, $P < \mathbf{0.01^*}$).

Table 4.1: The 6 reactivity variables measured in this experiment, how each variable was derived and the aspect of anxiety it represents. The prediction column refers to what we would expect to happen to the variable if a bird displayed an anxiety-like state. Hence, this was what we predicted for the difference between the call and no-call trials, and for the birds that had experienced greater early-life adversity.

Reactivity Variable	Calculation of Variable	Measure of Anxiety	Prediction
Rate of Movement	Total number of movements per minute	General Activity	↑
Duration Wall	Duration of time spent on the walls (s)	Escape Motivation	↑
Latency to Move	Latency to move body (s)	Initial Reactivity and Risk Assessment	↑
Average Use	Total duration of time using the resource / number of resource use bouts	Resource Motivation	↓
Head-Change Rate	Total number of head change movements / total duration using the resource	Vigilance (Experiment 1 only)	↑
Head-Up Proportion	Length of time head-up / total duration using the resource	Vigilance (Experiment 1 only)	↑

One hundred and twenty videos were scored in total, with 10% of these re-scored by the same experimenter as a measure of intra-observer reliability. All variables measured showed very strong correlations between the two scoring times, measured using intra-class correlation coefficients using the equation described by Lessells & Boag (1987) (rate of movement: ICC = 1, $F(11,12) = 4591$; duration of time spent on wall: ICC = 0.99, $F(11,12) = 358$; latency to move: ICC = 1, $F(11,12) = 584995$; average resource duration: ICC = 1, $F(11,12) = 54310$; head change rate: ICC = 0.99, $F(7,8) = 174$; head-up proportion: ICC = 0.94, $F(7,8) = 34.2$).

4.2.4 Statistical Analysis

Anxiety-like behaviour was characterised by the following variables in both experiments: a) rate of movement (total number of movements per minute), b) duration of time spent on the wall, c) latency to move, and d) average resource use (duration of time using the resource / number of resource use bouts). In experiment 1, e) head-change rate (total number of head change movements / duration using the food bowl) and f) head-up proportion (length of time with head spent up / duration using the food bowl) were also analysed.

The effect of potentially confounding variables that were not balanced between treatment groups (body condition and sex) were assessed by testing each as a sole predictor on each anxiety variable. There were no effects of sex or body condition on any reactivity variable and were therefore excluded from further analyses. Where the outcome variable was rate of movement or duration of time spent on the wall, data were log+1 transformed to improve model fit. Latency to move was also added as a covariate in these variables to control for individual differences in time spent active. Where analysing the effect of developmental treatments, the base model for each bias variable had fixed effects of Amount, Effort as well as the interaction between the two treatments. Where analysing the effect of DTA, the base model included a fixed effect of DTA between day 5 and day 56 post-hatch. Where analysing the effect of CORT variables (measured in chapter 3: experiment 1), fixed effects included baseline CORT, peak CORT and ΔCORT. In all models, data points are values from a single trial, with random effects of bird and natal nest included to account for non-independent repeated measures.

4.3 Results

4.3.1 Sex and Body Condition

Mass and body condition were significantly correlated ($r = 0.98$, $P <$ **0.01***). Males had a significantly higher body condition than females (mean males 7.47, mean females 3.23, Table 4.2) however there

were no significant effects of either treatment on body condition (mean LE 6.96, mean LH 3.00, mean PE 6.32, mean PH 5.03 , Table 4.2).

Table 4.2: Output from linear mixed effect models predicting body condition from sex and experimental treatments

Model Number	AICc	Dependent Variable	Fixed Predictors	LRT	*P*	n
1	201.7	Body Condition	Sex	4.32	0.04*	30
			Amount*Effort	0.43	0.51	
2	198.7	Body Condition	Sex	4.63	0.03*	30
			Amount	2.25	0.13	
			Effort	0.04	0.84	

4.3.2 Behavioural Variables

Figure 4.2 shows how the behavioural variables used to measure anxiety-like behaviour in experiment 1 and experiment 2 correlated. The only significant correlation identified was a negative relationship between head change rate and use of the resource in experiment 1 (r = -0.57, *P* < **0.05***).

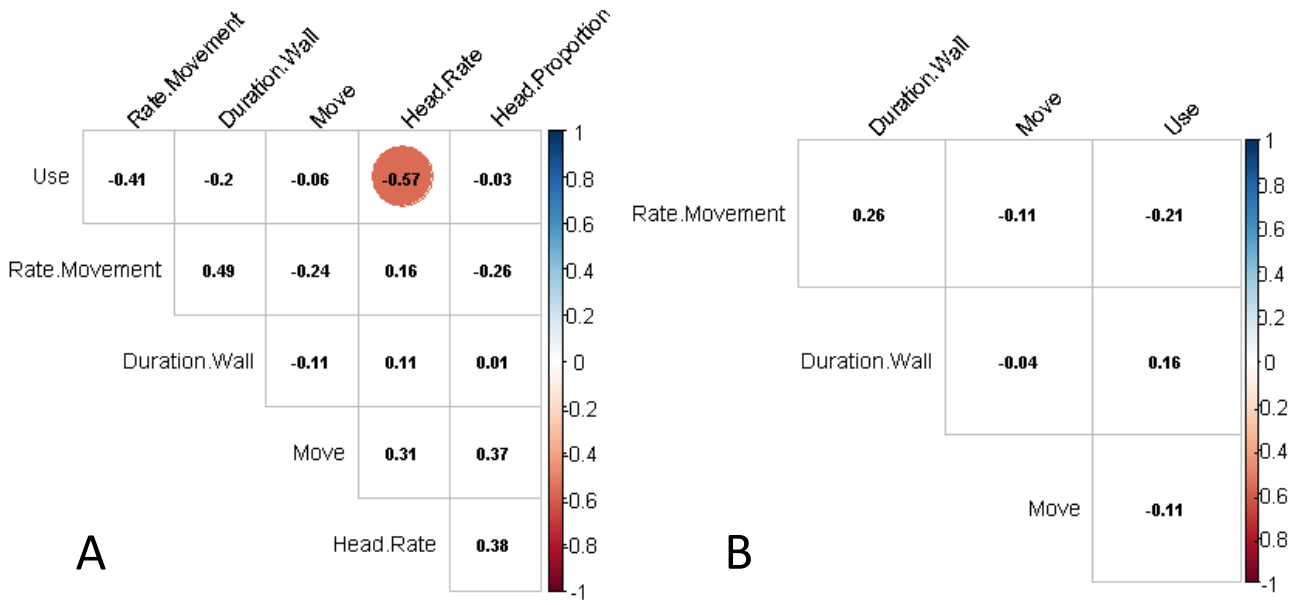

Figure 4.2: Correlation matrices for variable of anxiety in A) experiment 1 and B) experiment 2. A coloured circle represents a significant correlation, with the colour indicating the direction of the relationship (blue = positive, red = negative).

4.3.3 Familial Effects

To examine the effects of natal nest (including genetic, very early-life environmental or parental quality effects) on each reactivity variable, a variance components analysis was used. The mean of each reactivity variable was taken across the 4 days (2 days in the cases of head-change rate and head-up proportion). A model was fitted with an intercept and a random effect for natal nest for each reactivity variable using restricted ML estimation. Natal nest accounted for 0% of the variation in rate of movement, 3% of the variation in duration of time spent on the wall, 60% of the variation in latency to move, 37% for average resource duration, 4% for head change rate and 6% for head up proportion (Figure 4.3).

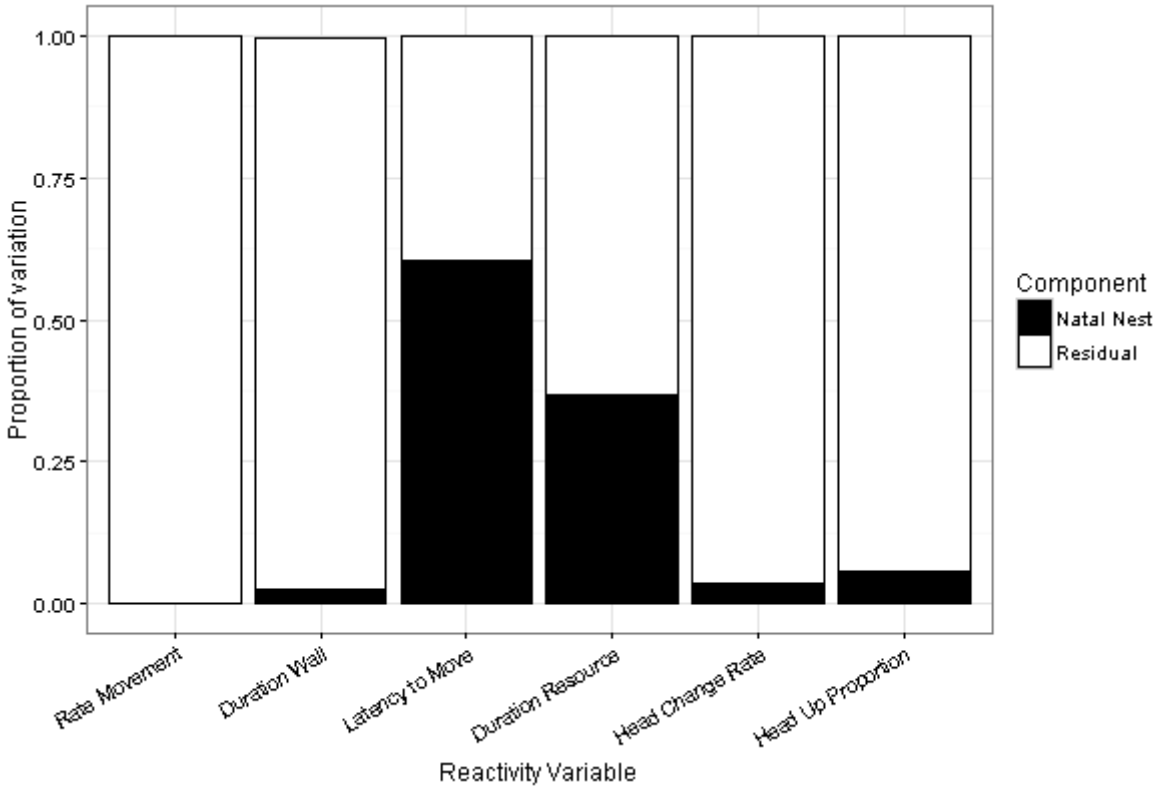

Figure 4.3: The familial and residual components of variation for each of the reactivity measures in the starling cohort.

4.3.4 Experiment 1

Experiment 1 refers to the behavioural trials in which a bowl of *ad lib* food was returned.

4.3.4a Developmental Treatment

We ran a series of mixed effects models predicting each reactivity variable (rate of movement, duration of time on the wall, latency to move, average resource use, head change rate and head up proportion) from the experimental treatments (Amount, Effort) as both independent and interacting

factors (Table 4.3). Each reactivity variable was tested with a three-way interaction of Amount*Effort*Stimulus (Call vs No-Call) which was dropped to two-way interactions if non-significant. These were again dropped to main effect models if non-significant. Throughout, stimulus had a significant effect on every reactivity variable, with exposure to the distress call leading to higher rates of movement, longer time spent on the wall, longer latencies to move, less time using the resource, and more vigilant behaviour (greater head transition rate and greater head up proportion. (Table 4.3; Figure 4.4).

In experiment 1, there were a number of significant associations between developmental treatments and reactivity variables. The significant developmental manipulation effects were as follows. There was a significant Amount*Stimuli interaction on latency to move (Figure 4.5a, Model 10-11). Lean birds were significantly slower than Plenty birds to start moving when exposed to the distress call. Effort had a main effect on head change rate, with Hard birds showing a greater rate of head-up/head-down changes (Figure 4.5b, Model 17). Finally, there was a significant three-way interaction between Amount, Effort and stimulus on head-up proportion (Figure 4.5c, Model 18). Lean-Hard and Plenty-Easy head-up proportions were consistent across the two stimuli, however Lean-Easy and Plenty-Hard birds showed less vigilant behaviour in the no call trials. In all other cases, none of the developmental effects were significant.

Table 4.3: Output from linear mixed effect models predicting stress reactivity variables from experimental treatments (Amount, Effort) and the acoustic stimulus in experiment 1. Where abbreviated, A = Amount, E = Effort, S = Stimulus. A * represents an interaction term. Move = Latency to move (as explained in 4.2.4)

Model Number	AICc	Reactivity Variable	Fixed Predictors	LRT	*P*	B (± SE)	N
3	96.1	Rate Movement	Move	2.65	0.10	0.73 (0.23)	60
			A*E*S	0.29	0.59	0.18 (0.36)	
4	93.2	Rate Movement	Amount*Effort	3.68	0.05~	-0.45 (0.24)	60
			Amount*Stimulus	0.32	0.57	-0.10 (0.19)	
			Effort*Stimulus	0.07	0.79	-0.05 (0.19)	
5	88.9	Rate Movement	Stimulus	8.01	< 0.01*	-0.27 (0.09)	60
			Amount	2.09	0.15	0.18 (0.12)	
			Effort	0.01	0.90	0.01 (0.12)	
6	235.5	Duration Wall	Move	6.55	0.01*	<0.01 (<0.01)	60
			A*E*S	1.64	0.20	1.37 (1.15)	

7	234.0	Duration Wall	Move	7.27	< 0.01*	<0.01 (<0.01)	60
			Amount*Effort	0.86	0.35	-0.86 (0.98)	
			Amount*Stimulus	0.17	0.68	-0.23 (0.60)	
			Effort*Stimulus	0.95	0.33	0.53 (0.58)	
8	227.3	Duration Wall	Move	9.31	< 0.01*	<0.01 (<0.01)	60
			Stimulus	20.77	< 0.01*	-1.67 (0.33)	
			Amount	0.36	0.55	-0.28 (0.49)	
			Effort	0.93	0.34	0.45 (0.48)	
9	845.5	Latency to Move	A*E*S	0.94	0.33	-202.4 (222.7)	60
10	843.4	Latency to Move	Amount*Effort	1.36	0.24	123.9 (112.4)	60
			Amount*Stimulus	4.40	0.04*	224.3 (111.3)	
			Effort*Stimulus	0.18	0.67	-44.3 (111.1)	
11	839.3	Latency to Move	Amount*Stimulus	4.29	0.04*	224.3 (110.8)	60
			Effort	0.48	0.49	37.13 (55.7)	
12	605.1	Resource Use	A*E*S	0.57	0.45	18.90 (26.85)	60
13	602.6	Resource Use	Amount*Effort	3.83	0.05~	40.72 (21.05)	60
			Amount*Stimulus	0.11	0.74	4.20 (13.40)	
			Effort*Stimulus	0.25	0.62	-6.30 (13.39)	
14	598.5	Resource Use	Stimulus	13.02	< 0.01*	25.36 (6.53)	60
			Amount	0.49	0.48	7.63 (11.20)	
			Effort	< 0.01	0.98	-0.29 (11.19)	
15	-38.4	Head Change Rate	A*E*S	0.08	0.78	-0.03 (0.11)	60
16	-41.4	Head Change Rate	Amount*Effort	0.55	0.46	-0.08 (0.11)	60
			Amount*Stimulus	1.17	0.28	0.06 (0.05)	
			Effort*Stimulus	0.25	0.61	-0.03 (0.05)	
17	-47.7	Head Change Rate	Stimulus	19.26	< 0.01*	-0.13 (0.03)	60
			Amount	0.01	0.91	< 0.01 (0.05)	
			Effort	3.91	0.05*	0.11 (0.05)	
18	-71.3	Head Up Proportion	A*E*S	4.58	0.03*	-0.17 (0.08)	60

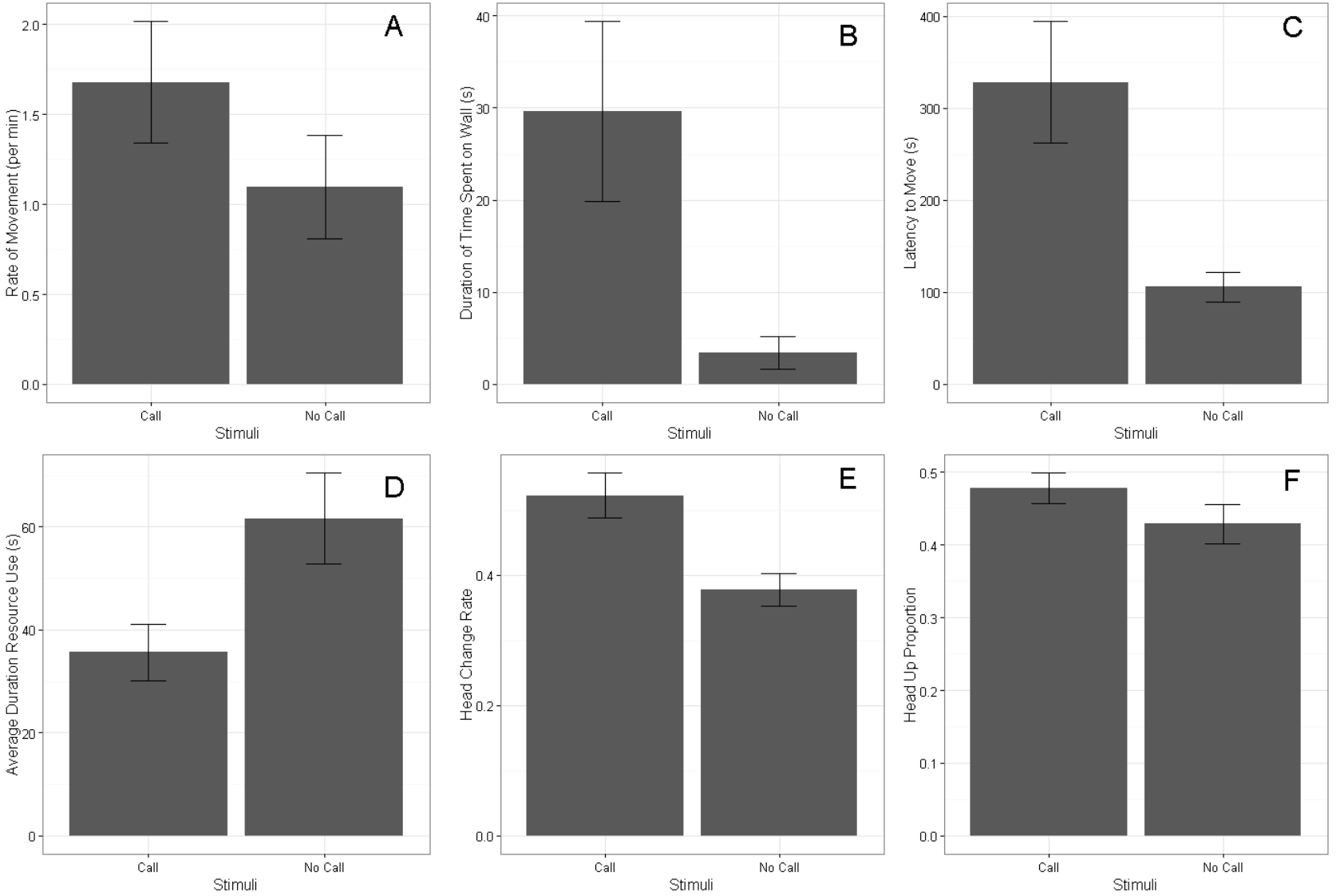

Figure 4.4: The effect of the acoustic stimulus on the mean of rate of movement (A), duration of time spent on the wall (B), latency to move (C), average duration of time spent using the resource (D), head change rate (E) and head up proportion (F) in experiment 1. Error bars represent one standard error of the mean (n=60). All graphs show raw data. Stimulus had a significant effect on all 6 variables.

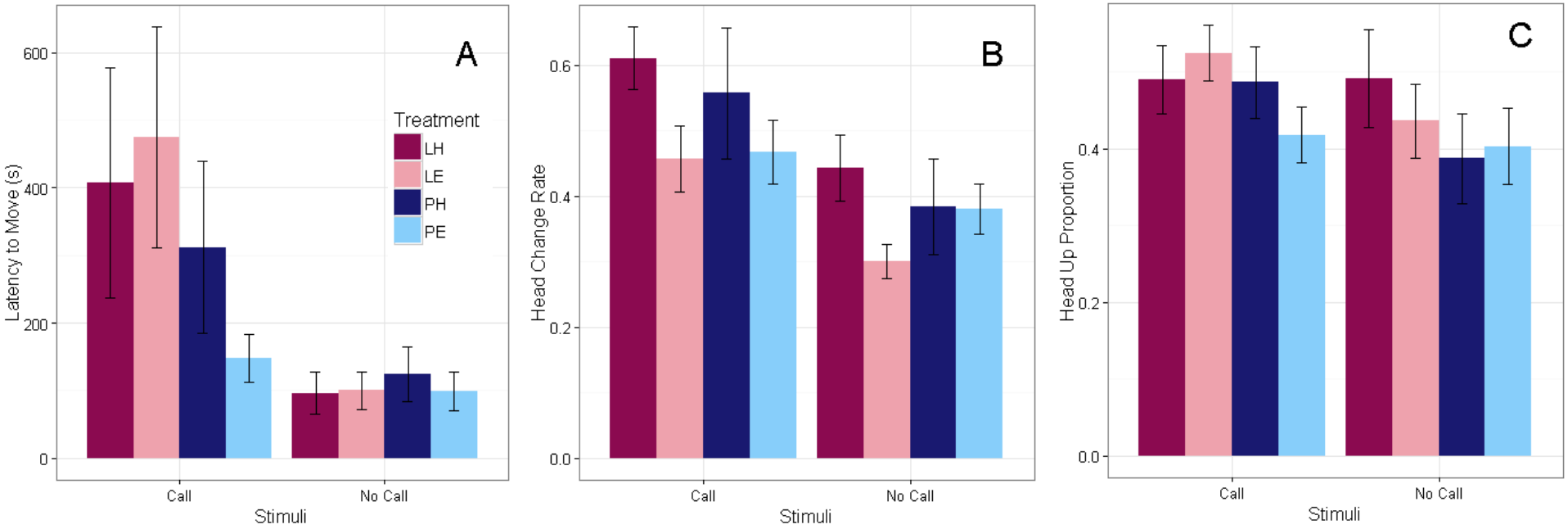

Figure 4.5: The effect of developmental treatments and stimulus on A) the mean of latency to move, B) head-change rate and C) head-up proportion in experiment 1. Treatment groups were LH (Lean-Hard), LE (Lean-Easy), PH (Plenty-Hard) and PE (Plenty-Easy). Error bars represent one standard error of the mean (n = 60).

4.3.4b Developmental Telomere Attrition

Experimental treatments were replaced with DTA (Table 4.4). There were no DTA effects on any reactivity variables in experiment 1.

Table 4.4: Output from linear mixed effect models predicting stress reactivity variables from DTA and the acoustic stimulus in experiment 1. A * represents an interaction term. Move = Latency to move (as explained in 4.2.4)

Model Number	AICc	Reactivity Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
19	85.9	Rate Movement	Move	4.79	0.03*	<0.01 (<0.01)	52
			DTA*Stimulus	0.03	0.85	0.08 (0.43)	
20	83.1	Rate Movement	Move	4.92	0.02*	<0.01 (<0.01)	52
			Stimulus	11.69	< 0.01*	-0.42 (0.11)	
			DTA	0.07	0.79	- 0.09 (0.35)	
21	195.7	Duration Wall	Move	7.67	< 0.01*	<0.01 (<0.01)	52
			DTA*Stimulus	0.49	0.48	-0.87 (1.30)	
22	193.3	Duration Wall	Move	8.88	< 0.01*	<0.01 (<0.01)	52
			Stimulus	18.55	< 0.01*	-1.71 (0.35)	
			DTA	0.02	0.90	-0.15 (1.03)	
23	736.3	Latency to Move	DTA*Stimulus	2.53	0.11	422.2 (272.9)	52
24	736.1	Latency to Move	Stimulus	12.23	< 0.01*	-241.0 (66.2)	52
			DTA	0.80	0.37	-133.0 (152.5)	
25	522.4	Resource Use	DTA*Stimulus	0.28	0.60	14.66 (28.95)	52
26	520.0	Resource Use	Stimulus	14.50	< 0.01*	29.62 (6.96)	52
			DTA	0.53	0.47	20.45 (29.79)	
27	-34.5	Head Change Rate	DTA*Stimulus	0.38	0.54	-0.07 (0.12)	52
28	-36.8	Head Change Rate	Stimulus	18.31	< 0.01*	-0.15 (0.03)	52
			DTA	1.20	0.27	-0.14 (0.13)	
29	-59.8	Head Up Proportion	DTA*Stimulus	0.12	0.73	-0.03 (0.09)	52
30	-62.4	Head Up Proportion	Stimulus	4.75	0.03*	-0.05 (0.02)	52
			DTA	0.65	0.42	-0.08 (0.10)	

4.3.4c CORT

Table 4.5 shows the results of testing each reactivity variable against CORT variables (baseline CORT, peak CORT and ΔCORT – a measure of HPA negative feedback) measured in chapter 3. Peak CORT had no effect on any reactivity measures in experiment 1 (Table 4.5), however there were associations of baseline CORT and ΔCORT with anxiety-like behaviour. There was a stimulus by baseline CORT interaction on duration of time spent using the resource (Table 4.5, Model 37-38; Figure 4.6). Birds with higher baseline CORT spent longer eating when not exposed to the auditory stimulus, however this was not seen after the distress call. Baseline CORT levels had a significant association with latency to move (Table 4.5, Model 36; Figure 4.7a) and head change rate (Table 4.5, Model 40; Figure 4.7b), with higher circulating CORT predicting longer freezing reactions and a greater head change rate. ΔCORT also affected anxiety-like behaviour, with higher ΔCORT (and therefore potentially better negative feedback) associated with more head transition changes (Table 4.5, Model 42; Figure 4.7c) and less time spent using the food resource (Table 4.5, Model 38, Figure 4.7d).

Table 4.5: Output from linear mixed effect models predicting stress reactivity variables from CORT variables and the acoustic stimulus in experiment 1. Cort0 = baseline CORT, Peak = Peak Cort, Delta = ΔCORT. A * represents an interaction term. Move = Latency to move (as explained in 4.2.4)

Model Number	AICc	Reactivity Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
31	92.7	Rate of Movement	Move	8.19	< 0.01*	< 0.01 (0.01)	60
			Cort0*Stimulus	3.47	0.06~	-0.10 (0.06)	
			Peak*Stimulus	0.42	0.52	< 0.01 (0.02)	
			Delta*Stimulus	2.97	0.08~	-0.03 (0.02)	
32	89.4	Rate of Movement	Move	4.45	0.03*	< 0.01 (0.01)	60
			Stimulus	12.39	< 0.01*	-0.38 (0.10)	
			Cort0	1.51	0.22	- 0.06 (0.05)	
			Peak	< 0.01	0.99	< 0.01 (0.01)	
			Delta	0.74	0.39	0.02 (0.02)	
33	235.4	Duration Wall	Move	5.69	0.02*	< 0.01 (0.01)	60
			Cort0*Stimulus	0.49	0.48	-2.15 (0.90)	
			Peak*Stimulus	1.39	0.24	0.06 (0.06)	
			Delta*Stimulus	0.99	0.32	- 0.06 (0.06)	
34	228.4	Duration Wall	Move	5.11	0.02*	< 0.01 (0.01)	60
			Stimulus	17.25	< 0.01*	-1.56 (0.34)	
			Cort0	0.43	0.51	-0.11 (0.17)	
			Peak	< 0.01	0.92	< 0.01 (0.05)	

			Delta	2.27	0.13	0.07 (0.05)	
35	843.0	Latency to Move	Cort0*Stimulus	1.78	0.18	-50.21 (40.07)	60
			Peak*Stimulus	0.10	0.75	-3.32 (11.09)	
			Delta*Stimulus	2.79	0.09~	-19.00 (12.04)	
36	840.2	Latency to Move	Stimulus	13.60	< 0.01*	-222.81 (58.78)	60
			Cort0	4.91	0.03*	47.80 (21.64)	
			Peak	0.16	0.69	2.61 (6.16)	
			Delta	1.92	0.17	10.07 (7.13)	
37	598.0	Duration Resource	Cort0*Stimulus	4.19	0.04*	9.08 (4.65)	60
			Peak*Stimulus	0.02	0.88	-0.18 (1.24)	
			Delta*Stimulus	0.43	0.43	-0.99 (1.34)	
38	592.8	Duration Resource	Cort0*Stimulus	4.44	0.04*	8.80 (4.27)	60
			Peak	0.06	0.80	-0.27 (1.06)	
			Delta	6.01	0.01*	-3.16 (1.27)	
39	-41.8	Head Change Rate	Cort0*Stimulus	0.01	0.91	< 0.01 (0.02)	60
			Peak*Stimulus	0.83	0.36	< 0.01 (0.01)	
			Delta*Stimulus	0.01	0.91	< 0.01 (0.01)	
40	-49.5	Head Change Rate	Stimulus	19.48	< 0.01*	-0.13 (0.03)	60
			Cort0	4.14	0.04*	0.04 (0.02)	
			Peak	1.14	0.29	< 0.01 (0.01)	
			Delta	5.02	0.03*	0.01 (0.01)	
41	-68.6	Head Up Proportion	Cort0*Stimulus	0.76	0.38	0.01 (0.02)	60
			Peak*Stimulus	1.59	0.31	< 0.01 (0.01)	
			Delta*Stimulus	0.57	0.45	< 0.01 (0.01)	
42	-75.4	Head Up Proportion	Stimulus	5.06	0.02*	-0.05 (0.02)	60
			Cort0	0.06	0.80	< 0.01 (0.01)	
			Peak	0.26	0.61	< 0.01 (0.01)	
			Delta	2.03	0.15	< 0.01)	

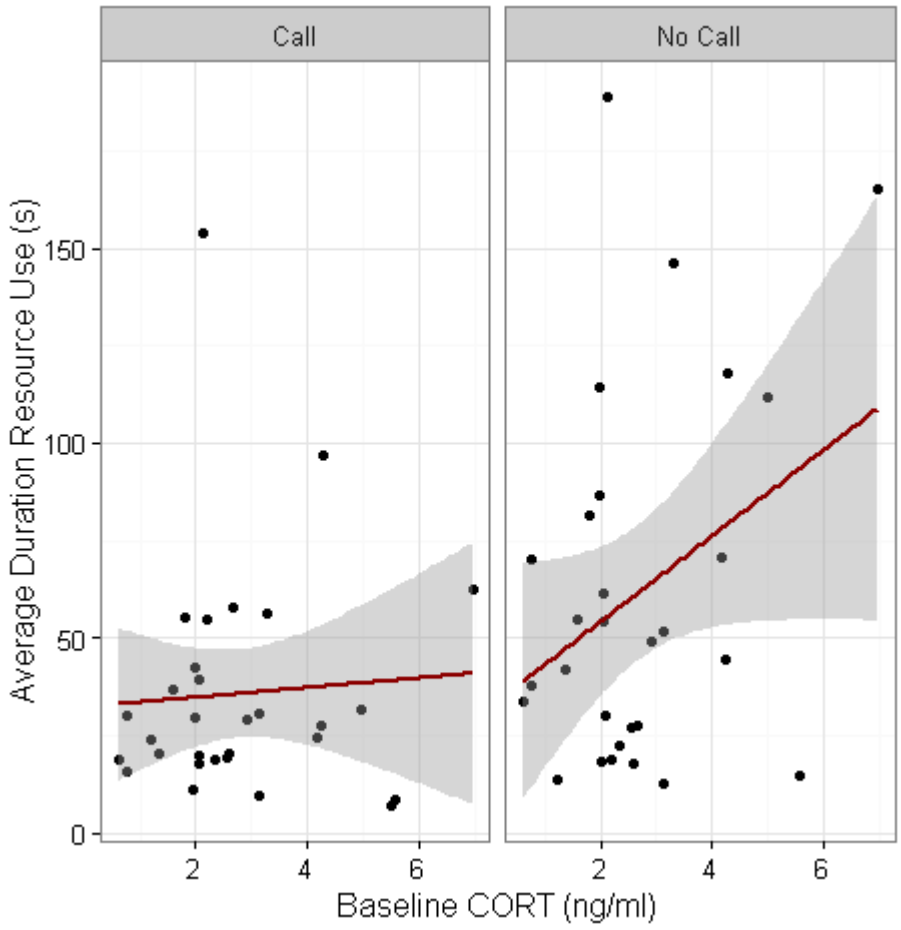

Figure 4.6: The effect of baseline CORT and acoustic stimulus on average duration resource use in experiment 1.

4.3.4d Summary of Experiment 1

Experiment 1 (testing anxiety-like behaviour in response to an alarm call, in the context of a food resource being returned) showed effects of both developmental treatments on reactivity. First, Lean birds were significantly slower than Plenty birds to start moving when exposed to the distress call (Figure 4.5a, Model 10-11). Second, Hard birds showed a greater rate of head-up/head-down changes (Figure 4.5b, Model 17). Finally, there was a significant three-way interaction between Amount, Effort and stimulus on head-up proportion (Figure 4.5c, Model 18). Experiment 1 also showed associations of CORT with reactivity. Birds with higher baseline CORT spent longer eating when not exposed to the auditory stimulus, however this was not seen after exposure to the distress call (Table 4.5, Model 37-38; Figure 4.6). Baseline CORT levels also had a significant independent association with latency to move and head change rate (Table 4.5, Model 36, Model 40; Figure 4.7a-b), with higher circulating CORT predicting longer freezing reactions and a greater head change rate. Finally, higher ΔCORT was associated with more head transition changes (Table 4.5, Model 42; Figure 4.7c) and less time spent using the food resource (Table 4.5, Model 38, Figure 4.7d).

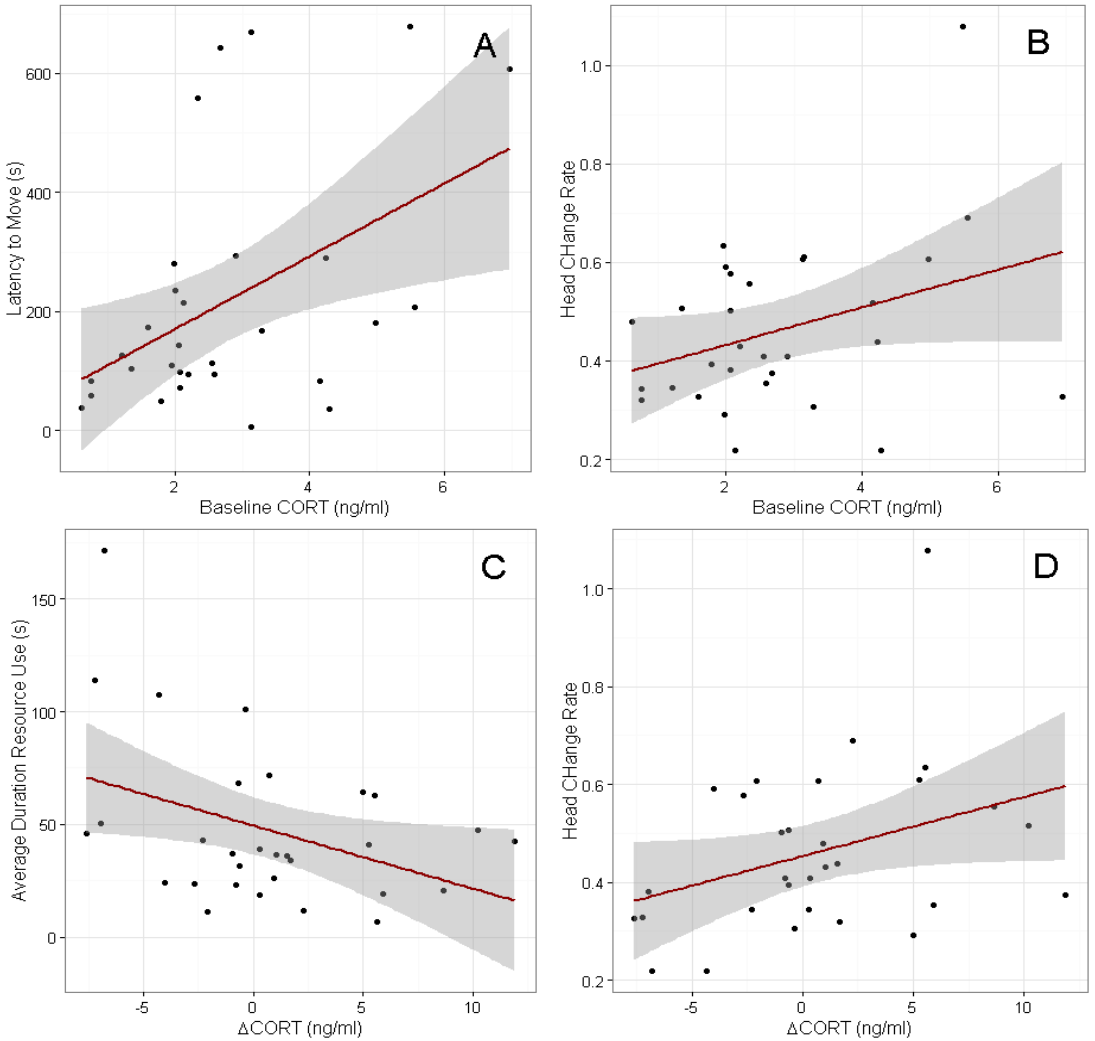

Figure 4.7: The effect of baseline CORT (a-b) and ΔCORT on several stress reactivity variables in experiment 1. A) Baseline CORT on latency to move. B) Baseline CORT on head-change rate. C) ΔCORT on average duration resource use and D) ΔCORT on head-change rate. ΔCORT is the change in CORT between Time 15 and Time 30 where a positive value indicates a reduction in CORT.

4.3.5 Experiment 2

Experiment 2 refers to the behavioural trials in which a water bath was returned.

4.3.5a Developmental Treatment

We ran a series of mixed effects models predicting each reactivity variable (rate of movement, duration of time on the wall, latency to move, average resource use, head change rate and head up proportion) from the experimental treatments (Amount, Effort) as both independent and interacting factors (Table 4.6). There were no developmental treatment effects on any reactivity variables in experiment 2. Stimulus had a significant effect on latency to move (Figure 4.8c), but none of the other reactivity variables (Figure 4.8a-b, d). Similar to experiment 1, birds were slower to begin moving in response to the distress call than when they had not heard the call (Table 4.6, Model 51).

Table 4.6: Output from linear mixed effect models predicting stress reactivity variables from experimental treatments (Amount, Effort) and the acoustic stimulus in experiment 2. Where abbreviated, A = Amount, E = Effort, S = Stimulus. A * represents an interaction term. Move = Latency to move (as explained in 4.2.4)

Model Number	AICc	Reactivity Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
43	109.1	Rate Movement	Move	1.55	0.21	1.84 (0.23)	60
			A*E*S	0.04	0.84	-0.07 (0.40)	
44	104.5	Rate Movement	Amount*Effort	< 0.01	0.96	0.02 (0.33)	60
			Amount*Stimulus	0.17	0.68	-0.08 (0.20)	
			Effort*Stimulus	< 0.01	0.96	-0.01 (0.20)	
45	96.4	Rate Movement	Stimulus	1.24	0.27	-0.10 (0.10)	60
			Amount	2.49	0.11	0.25 (0.16)	
			Effort	1.88	0.17	-0.22 (0.16)	
46	253.4	Duration Wall	Move	0.20	0.65	<0.01 (<0.01)	60
			A*E*S	0.03	0.87	0.18 (1.26)	
47	247.5	Duration Wall	Amount*Effort	0.43	0.51	-0.73 (1.18)	60
			Amount*Stimulus	0.86	0.35	0.54 (0.62)	
			Effort*Stimulus	1.15	0.28	0.63 (0.62)	
48	241.6	Duration Wall	Stimulus	1.25	0.26	-0.34 (0.31)	60
			Amount	0.24	0.62	0.27 (0.58)	
			Effort	0.58	0.45	-0.43 (0.58)	
49	806.8	Latency to Move	A*E*S	0.36	0.55	-86.44 (154.69)	60
50	804.2	Latency to Move	Amount*Stimulus	1.20	0.27	80.00 (77.1)	60
			Amount*Effort	0.21	0.64	34.5 (79.2)	

			Effort*Stimulus	0.18	0.67	-30.6 (76.9)	
51	797.4	Latency to Move	Stimulus	7.11	< 0.01*	-102.0 (38.2)	60
			Amount	0.47	0.49	-25.7 (38.6)	
			Effort	0.85	0.36	34.6 (38.6)	
52	477.3	Resource Use	A*E*S	1.05	0.30	-17.26 (18.13)	60
53	475.1	Resource Use	Amount*Effort	0.66	0.42	7.27 (9.47)	60
			Amount*Stimulus	0.31	0.57	-4.75 (9.08)	
			Effort*Stimulus	0.09	0.77	2.51 (9.04)	
54	467.4	Resource Use	Stimulus	< 0.01	0.94	0.35 (4.44)	60
			Amount	0.23	0.63	-2.07 (4.51)	
			Effort	1.88	0.17	6.02 (4.50)	

4.3.5b Developmental Telomere Attrition

Developmental treatments were replaced with DTA (Table 4.7). There was a significant interaction between DTA and stimulus on latency to move and on resource use (Table 4.7, Model 59-60). Birds with greater amounts of telomere attrition took longer to move when exposed to the distress call, however there was no such relationship in the no call condition (Figure 4.9a). Finally, birds with more telomere attrition bathed significantly less when exposed to the call and significantly more in the no call condition than birds with less DTA (Figure 4.9b).

Table 4.7: Output from linear mixed effect models predicting stress reactivity variables from DTA and the acoustic stimulus in experiment 2. A * represents an interaction term.

Model Number	AICc	Reactivity Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
55	92.3	Rate Movement	Move	1.79	0.18	<0.01 (<0.01)	52
			DTA*Stimulus	0.13	0.72	0.15 (0.45)	
56	88.7	Rate Movement	Stimulus	0.20	0.65	-0.04 (0.10)	52
			DTA	0.01	0.91	-0.04 (0.41)	
57	217.0	Duration Wall	Move	< 0.01	0.96	<0.01 (<0.01)	52
			DTA*Stimulus	0.20	0.66	0.67 (1.58)	
58	211.7	Duration Wall	Stimulus	0.73	0.39	-0.29 (0.35)	52
			DTA	0.31	0.58	-0.69 (1.28)	
59	691.0	Latency to Move	DTA*Stimulus	4.94	0.03*	362.4 (161.9)	52
60	402.4	Resource Use	DTA*Stimulus	4.56	0.03*	-42.81 (20.37)	52

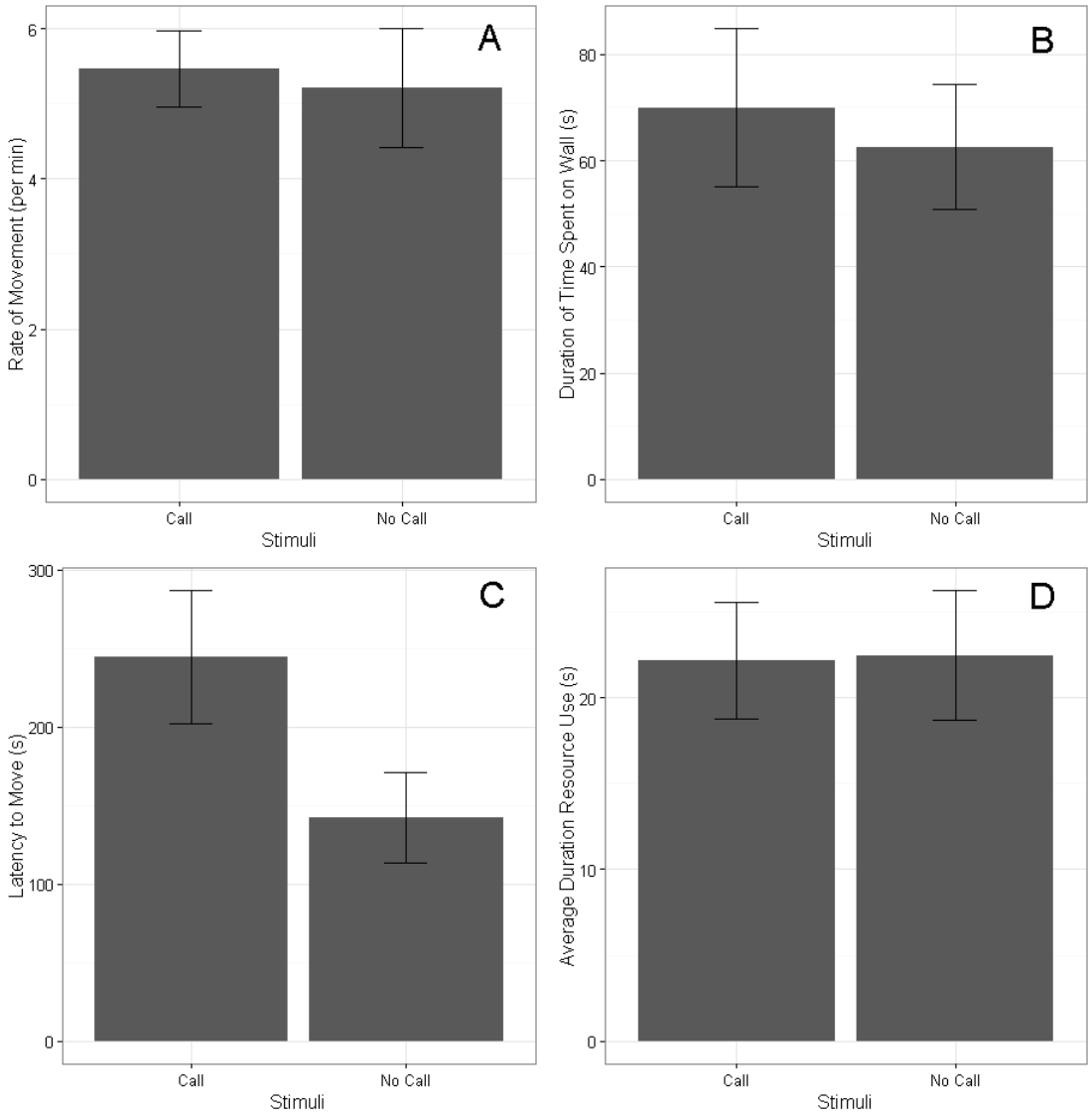

Figure 4.8: The mean effects of the acoustic stimulus on rate of movement (A), duration of time spent on the wall (B), latency to move (C) and average duration of time spent using the resource (D) in experiment 2. Error bars represent one standard error of the mean. All graphs show raw data. Stimulus had a significant effect on latency to move (Figure 5c).

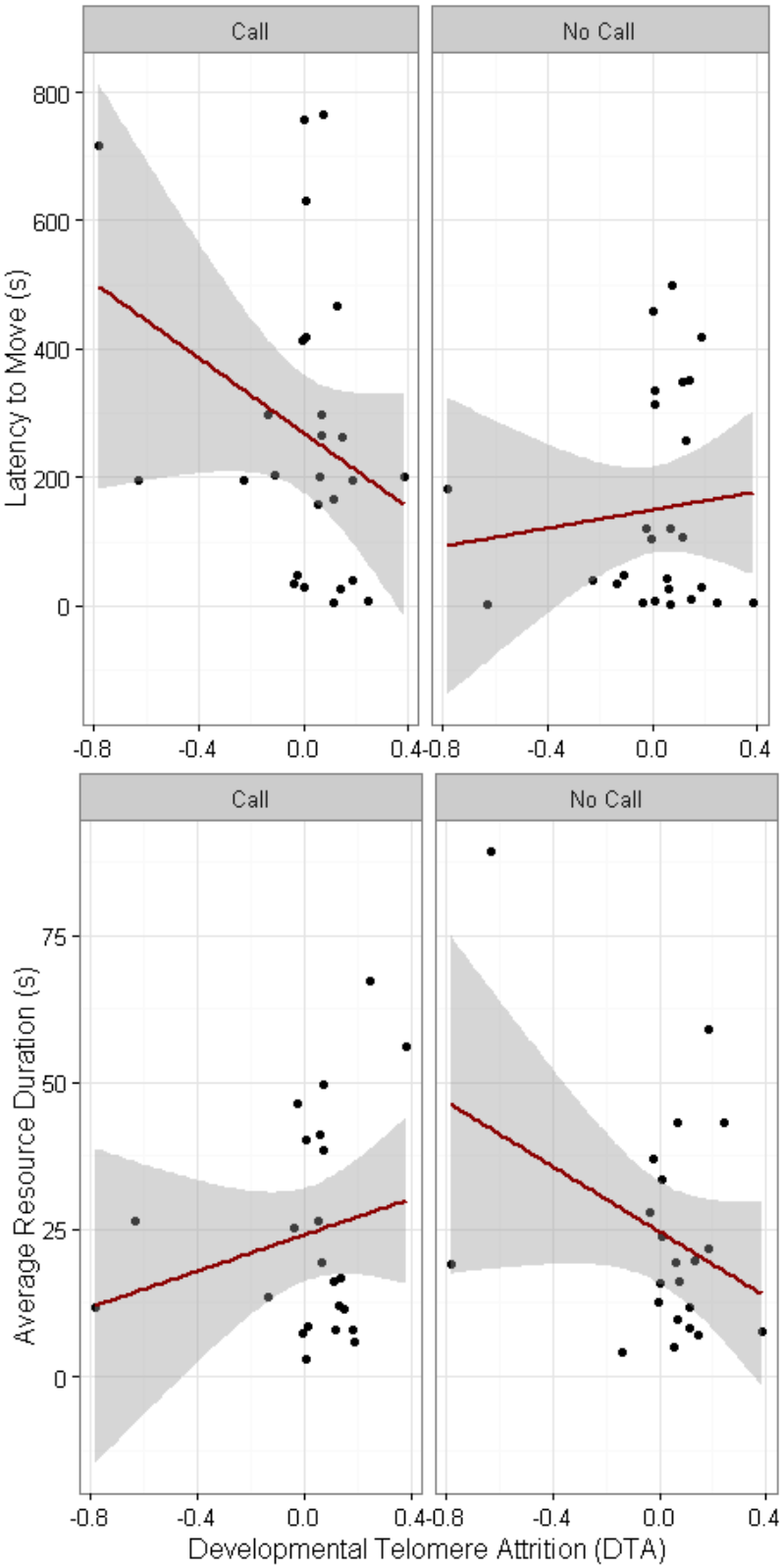

Figure 4.9: A) Top panel – the effect of developmental telomere attrition on latency to move. B) Bottom panel - The effect of developmental telomere attrition on average duration spent using the resource, after exposure to the call and the no call trials in experiment 2. More negative DTA indicates greater attrition.

4.3.5c CORT

Table 4.8 shows the results of testing each reactivity variable against CORT variables (baseline CORT, peak CORT and ΔCORT – a measure of HPA negative feedback) measured in chapter 3. There was no effect of peak CORT or ΔCORT on any reactivity measure in experiment 2 (Table 4.8). Baseline CORT had a significant effect on latency to move (Table 4.8, Model 66) and also on rate of movement (Model 62). Higher levels of baseline CORT predicted lower overall rate of movement (Figure 4.10a) and also a higher latency to move (Figure 4.10b).

Table 4.8: Output from linear mixed effect models predicting stress reactivity variables from CORT variables and the acoustic stimulus in experiment 2. Cort0 = baseline CORT, Peak = Peak Cort, Delta = ΔCORT. A * represents an interaction term. Move = Latency to move (as explained in 4.2.4)

Model Number	AICc	Reactivity Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
61	106.9	Rate of Movement	Move	1.37	0.24	0.01 (<0.01)	30
			Cort0*Stimulus	0.01	0.91	0.14 (0.31)	
			Peak*Stimulus	1.40	0.24	-0.10 (0.07)	
			Delta*Stimulus	0.91	0.34	0.03 (0.02)	
62	98.1	Rate of Movement	Stimulus	1.24	0.27	-0.11 (0.10)	30
			Cort0	4.38	0.04*	-0.12 (0.06)	
			Peak	2.05	0.15	0.02 (0.02)	
			Delta	0.05	0.82	< 0.01 (0.02)	
63	250.6	Duration Wall	Move	0.05	0.82	0.01 (<0.01)	30
			Cort0*Stimulus	4.46	0.05~	0.44 (0.22)	
			Peak*Stimulus	1.92	0.17	-0.08 (0.06)	
			Delta*Stimulus	0.21	0.65	0.03 (0.06)	
64	243.5	Duration Wall	Cort0	0.09	0.76	0.34 (0.20)	30
			Peak	1.22	0.27	0.06 (0.06)	
			Delta	< 0.01	0.93	< 0.01 (0.06)	
			Stimuli	1.25	0.26		
65	797.0	Latency to Move	Cort0*Stimulus	3.54	0.06~	44.36 (24.91)	30
			Peak*Stimulus	0.63	0.43	-5.11 (6.89)	
			Delta*Stimulus	< 0.01	0.94	0.50 (7.49)	
66	791.9	Latency to Move	Stimulus	8.24	< 0.01*	-102.00 (35.63)	30
			Cort0	8.13	< 0.01*	39.41 (13.94)	
			Peak	2.65	0.10	-6.64 (4.03)	
			Delta	0.02	0.90	0.64 (5.12)	

67	477.0	Duration Resource	Cort0*Stimulus	0.84	0.36	-3.21 (3.78)	27
			Peak*Stimulus	1.06	0.30	0.81 (0.85)	
			Delta*Stimulus	0.10	0.75	0.29 (1.00)	
68	469.5	Duration Resource	Stimulus	0.03	0.86	0.75 (4.49)	27
			Cort0	1.99	0.16	2.71 (1.98)	
			Peak	0.83	0.36	-0.42 (0.47)	
			Delta	0.49	0.49	-0.41 (0.61)	

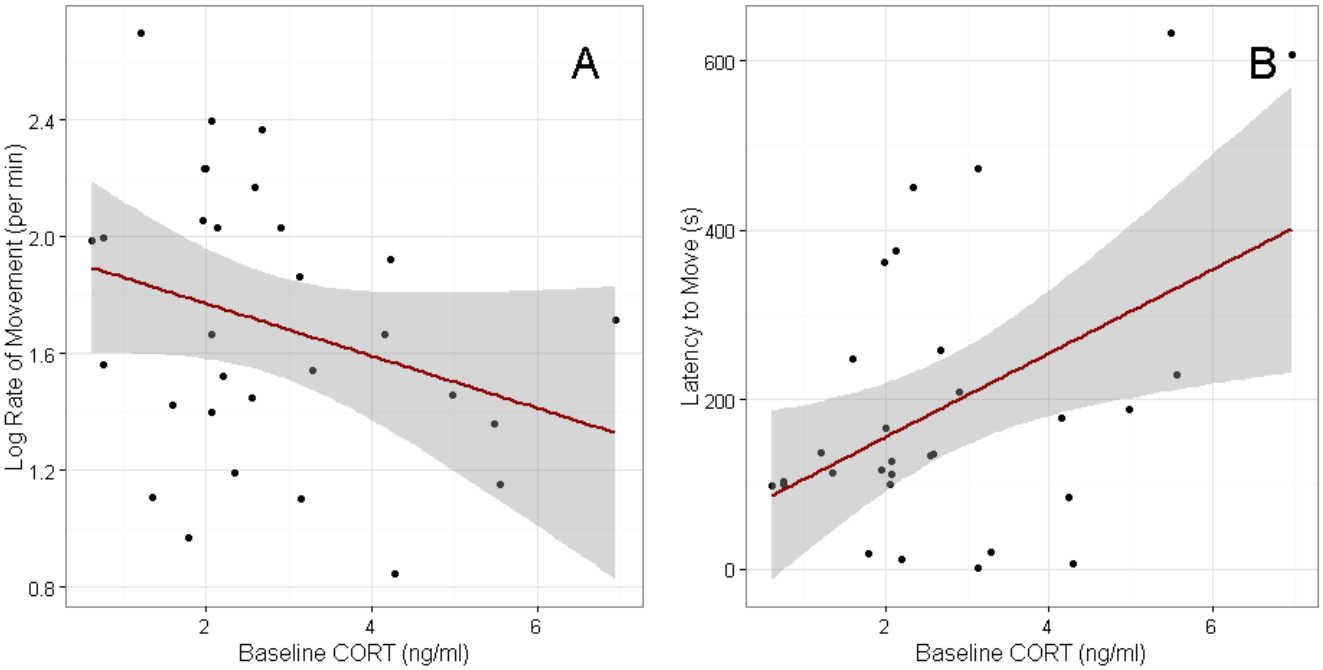

Figure 4.10: The effect of baseline CORT on A) rate of movement and B) latency to move. NB: Figure 4.10a has been plotted using logged data for clarity.

4.3.5d Summary of Experiment 2

To summarise, in experiment 2 we found that birds with greater amounts of telomere attrition took longer to move when exposed to the distress call, however there was no such relationship in the no call condition (Figure 4.9a). Birds with more DTA bathed significantly less when exposed to the call and significantly more in the no call condition than birds with less DTA (Figure 4.9b). We also showed associations of CORT with anxiety-like behaviour. Baseline CORT had a significant association with latency to move (Table 4.8, Model 66) and also with rate of movement (Model 62). Here, higher levels of baseline CORT predicted a lower rate of movement (Figure 4.10a) and also a longer latency to move (Figure 4.10b). There were no significant effects of developmental treatment on anxiety-like behaviour in experiment 2.

4.4 Discussion

4.4.1 Summary of Results

Using a cohort of European starlings, we investigated the efficacy of a distress call to create an anxious state in birds, and the effect of early-life adversity on this response. We tested a suite of behaviours that represented different aspects of anxiety with respect to the independent introduction of two resources, a bowl of *ad lib* food (experiment 1) and a water bath (experiment 2), both of which the birds had been deprived prior to testing. Anxiety-like responses were measured after exposure to a distress call or a period of silence as a control condition. We found that the distress call stimulus significantly increased anxiety-like behaviour throughout experiment 1, however we did not see these effects in the majority of behaviours tested in experiment 2.

We found significant effects of the developmental treatments in experiment 1 but not in experiment 2. First, latency to move was affected by Amount after exposure to the distress call. In these trials, Lean birds (birds who had been fed only 73% of *ad lib* during development) were significantly slower than Plenty birds (birds who had been fed *ad lib)* to begin moving. Second, Effort had a main effect on head-change rate, with Hard birds (birds who experienced increased begging effort) showing a greater rate of head-up/head-down changes than Easy birds (birds who had experienced less begging effort), regardless of stimulus exposure. Finally, there was a significant three-way interaction of Amount, Effort and acoustic stimulus on head-up proportion. This was a complicated result to interpret. Lean-Hard and Plenty-Easy head-up proportions were consistent across the two stimuli, however Lean-Easy and Plenty-Hard birds showed less vigilant behaviour in the no call trials. We found no effects of DTA in experiment 1, however DTA was a significant predictor of anxiety in experiment 2. After exposure to the distress call, birds with greater DTA had longer latencies to move and spent less time bathing.

The final aspect of this study examined how physiological data collected from this cohort, namely data collected about the HPA axis, correlated with anxiety-like behaviour. With CORT concentration data collected approximately 4 months prior to this study, we showed that baseline CORT and ΔCORT (but not peak CORT) were significantly associated with behaviour in both experiment 1 and experiment 2. In experiment 1, increased baseline CORT was associated with anxiety-like behaviour in terms of increased latency to move and head change rate. There was an interaction between baseline CORT and stimulus on average resource duration, with higher baseline CORT predicting longer use of the resource in the no call trials, but not after exposure to the distress call. Finally, ΔCORT had a relationship with head change rate and average duration of the resource in experiment 1. Birds with higher levels of ΔCORT, and therefore faster cessation of the stress response, spent less time eating and had a greater head change rate regardless of trial type. In experiment 2, baseline CORT affected

movement, with higher baseline CORT associated with a lower rate of movement and an increased latency to move.

4.4.2 Stimulus and Resource Differences

We predicted that anxiety-like behaviour would be increased by exposure to the distress call in both experiments. This prediction was met in experiment 1, with increases seen in rate of movement, time spent in peripheral locations, latency to move, displays of vigilant behaviour and decreases in time spent eating after experience of the distress call compared to no call trials. This is compelling evidence that a conspecific distress call can create an anxiety-like state in European starlings. In experiment 2, however, only latency to move was significantly increased by the distress call. There are several reasons why experimental differences may have occurred. Due to the ordering of the experiments, the distress call was not novel by experiment 2. However, it is unlikely that birds had habituated to the distress call after one presentation. Biologically important stimuli such as distress calls convey critical information necessary for survival and are unlikely to be ignored after a single event. In support of this, habituation to distress calls has been shown to be significantly slower than to sounds such as white noise and human voices (Thompson et al. 1968; Johnson et al. 1985). Second, birds had longer to habituate to individual cages and visual isolation by experiment 2 and this may have reduced underlying baseline anxiety. Finally, experimental differences in the response to the distress call could be due to perceived differences between the two resources tested.

We found that whilst the distress call increased latencies to move, there was no effect of the stimulus on other anxiety-like behaviours in experiment 2. Brilot & Bateson (2012) demonstrated that birds who were deprived of baths were more sensitive to threats and showed more anxiety-like behaviour when tested with a food bowl. Due to this, we predicted that the distress call would have a large effect when birds had been deprived of water baths. Brilot et al. (2009a) demonstrated that restricting access to water baths caused birds to alter escape flight patterns, by prioritising flight accuracy over flight speed, showing the importance of bathing in defensive behaviour. Our results could imply that, in experiment 2, birds were in a high state of anxiety, regardless of whether the acoustic stimulus was played, due to being deprived of water baths. Therefore there were no increases in anxiety-like behaviour when comparing results gained from the 'Call' trials to the 'No Call' trials.

It is important to clarify that this experimental design has not been able to test anxiety-like behaviour in a "stressed" and "non-stressed" condition. Whilst the 'No Call' trials were conducted with minimal disruption to the animals, the trials consisted of birds being exposed to sudden darkness (associated with experimenters entering the room to catch the animals) and of the experimenter putting their hands into birds' home cages. There will be individual differences in how stressful birds find this

process, and therefore the no call trial types should be interpreted as a baseline reaction with caution. Instead, we propose that this experiment has given insight into anxiety-like behaviour with respect to a mild stressor (no call condition) and a moderate stressor (call condition). It was also not possible to directly compare results from experiment 1 and experiment 2 due to order effects. In small sample sizes such as ours, maintaining power is crucial and would be further reduced with multiple cases of counter-balancing. Therefore, only stimulus was counter-balanced for practicality to maintain effective sample sizes. A similar experiment with a larger cohort of birds would allow more direct investigation into the differences between food and bath deprivation with respect to anxiety.

4.4.3 Developmental Treatment and Telomere Attrition

We found significant effects of the developmental treatments in experiment 1 (but not in experiment 2) with both Amount and Effort altering several behaviours linked to anxiety. We saw long-lasting effects of Amount on anxiety-like behaviour exhibited under threat as predicted, with Lean birds exhibiting more initial freezing behaviour after hearing the distress call. This is in accordance with previous studies concerning food availability. Prenatal and early-life dietary restriction in rats has been shown to increase anxiety-like behaviour (Jahng et al. 2007; Levay et al. 2008) with both leptin resistance and decreased serotonergic activity suggested as possible mechanisms explaining this. Interestingly, in experiment 1, Hard birds showed greater levels of vigilance (measured by head-up/head-down change rates) than Easy birds, regardless of exposure to the distress call. This demonstrates that our Effort treatment significantly increased baseline anxiety and therefore could permanently alter a bird's susceptibility to develop anxiety-related problems. The unpredictability of the early environment has been shown to be important in the development of anxiety in the non-human primate literature. Varying the amount of work each day that maternal macaques had to perform to gain food led to an increase in anxiety-like behaviour in offspring, demonstrated by more clinging behaviour in novel environments and less explorative behaviour (Andrews & Rosenblum 1994; Rosenblum & Paully 1984). Variable foraging demand paradigms have been shown to increase cerebrospinal fluid concentrations of corticotropin-releasing factor which, in turn, increases HPA activity (Coplan et al. 1996; Coplan et al. 2001). Unpredictability in food supply and increased begging effort experienced by Hard birds could also lead to more vigilant behaviour through similar mechanisms, although further physiological experiments are necessary to confirm this.

Unlike the developmental treatments, we found no effect of DTA on any reactivity variable in experiment 1, however more anxiety-like behaviour was seen in individuals with more DTA in experiment 2. First, we found that birds with more DTA took significantly longer to move after exposure to the distress call, but not in the 'No Call' condition. Second, DTA affected how birds used

the water bath with respect to the distress call. More DTA was associated with less time spent in the water bath after hearing the distress call, and this was compensated for in the no call trials. This increase in anxiety-like behaviour is reflected in the human literature, with shorter telomeres often reported in individuals suffering from anxiety disorders (Simon et al. 2006; Okereke et al. 2012; Needham et al. 2015). The mechanisms underlying DTA and the development of anxiety are still to be discovered, however studies such as ours provide evidence for the role of shorter telomeres in the development of mood disorders such as anxiety.

Previously, we have shown that developmental treatments had direct effects on DTA, with birds from more stressful treatments showing greater levels of DTA (Nettle et al. 2017; Chapter 2). Whilst our experiment met the prediction that more stressful treatments and greater DTA would increase anxiety-like behaviour, treatment effects were seen exclusively in experiment 1, and DTA effects in experiment 2. This implies that developmental treatments and DTA are having independent effects on anxiety-related mechanisms, as opposed to DTA being a by-product of the developmental manipulation. Bateson et al. (2015) also showed that, in starlings, DTA and early-life experience could have different effects on a measure associated with mood and emotion (cognitive bias, see chapter 5 for more details), even though early-life experience contributed to variation in DTA. We also found that developmental treatments and DTA did not affect all stress reactivity behaviours measured in this experiment. This is unsurprising as is it known that different aspects of anxiety exist and are therefore likely to be affected by different stressors (Gaalen et al. 2000; Levay et al. 2008; Kalinichev et al. 2002). Latency to move, however, was the only variable to be affected by early-life adversity and also by the distress call in both experiments. Our data therefore suggest that this measure is particularly important in the study of anxiety, and is particularly sensitive to early-life adversity.

4.4.4 CORT

We predicted that an increase in baseline CORT would be associated with more baseline anxiety. This prediction was met across both experiments with higher baseline CORT associated with longer latencies to move and increased vigilance regardless of trial type in experiment 1. In experiment 2, we found that baseline CORT was associated with a lower rate of movement, contrary to our predictions. However this can be explained by a few animals exhibiting a more passive coping strategy (freezing behaviour) when threatened by a predator (as opposed to an active strategy – flight and escape attempts). Therefore both an increase and decrease in rate of movement could be associated with more anxiety-like behaviour (Steimer 2002). We also found that baseline CORT associated with increases in anxiety exhibited under threat, with a CORT by stimulus interaction on the duration of time spent using the resource in experiment 1. Here, birds with higher CORT spent longer eating than

in the no call condition. Whilst we found no link between peak CORT and anxiety-like behaviour, we found associations of ΔCORT in experiment 1. More positive values of ΔCORT, potentially suggesting better negative feedback of the HPA axis, were associated with more anxiety-like behaviour in terms of duration of time spent eating and head-change rate. ΔCORT is a measure of CORT change between 15 minutes and 30 minutes post-stressor. Therefore, interpretation of this result is difficult as any behaviour elicited before this time period may not be mediated by ΔCORT. Alternatively, ΔCORT could be interpreted as a proxy of the timing of the CORT peak, with positive ΔCORT indicating that the peak has occurred before 30 minutes and negative ΔCORT indicating that this peak has yet to occur. Anxiety-like behaviour could be elicited by an individual's maximum CORT concentrations. This could explain why birds with increased ΔCORT (and therefore had already reached peak CORT levels) exhibited more anxiety-like behaviour than birds that had not yet reached peak CORT levels.

Whether or not high levels of CORT are anxiogenic is disputed in the literature. Many studies have shown that experimentally increasing circulating CORT can lead to anxiety-like behaviour (Fan et al. 2014; Mitra & Sapolsky 2008). However several experimental and correlational studies have suggested CORT to have anxiolytic effects (Albrecht et al. 2013; Heim & Nemeroff 2001). Our data suggests anxiety-inducing effects of increased baseline CORT and greater ΔCORT. Smythe et al. (1997) demonstrated that by blocking CORT from binding to mineralocorticoid receptors (MR) (a key component of the HPA negative feedback process), they were able to experimentally reduce anxiety-like behaviour. It is important to state that our physiological measures were not taken at the time of the behavioural study and are therefore purely correlational. We have previously shown that repeatability of baseline CORT and peak CORT is limited in our cohort (chapter 3) so a further measure of HPA reactivity at the time of this study would provide more accurate insight into the physiological response of the birds to the distress call.

4.4.5 Conclusion

We aimed to develop a behavioural-based test of anxiety using a starling distress call with respect to two introduced resources. Our results from experiment 1 confirmed that a distress call was sufficient to generate an anxiety-like state in European starlings. Whilst not directly comparable, we found that developmental treatments, DTA and CORT profiles did not have consistent effects on anxiety demonstrated by the birds over the two experiments. This suggests that resources play different roles when exposed to a predatory threat. It would be interesting to further explore the effects of food and bath deprival by testing behaviour in response to different anxiogenic stimuli such as novel environments.

We found that early-life adversity (both in terms of our developmental treatments and DTA) had long-lasting effects on a wide variety of anxiety-like behaviour with more early adversity associated with an increase in anxiety. Our results are of interest to human studies, with our data providing further evidence that early-life experience can cause permanent changes to behaviour that can leave individuals vulnerable to the development of anxiety disorders. Further to this, we have shown that measures of the HPA axis (increased circulating CORT in particular) are intrinsically linked with increased anxiety, suggesting that early-life experiences can directly affect the development of mood disorders through the disruption of the HPA axis. Interestingly, improvement in a measure of HPA axis feedback was shown to be anxiogenic, however further experiments specifically designed to test this hypothesis are needed. Our cohort of starlings is unique in several ways. This longitudinal cohort study allows us to take regular physiological and behavioural measures to assess how early-life adversity can lead to anxiety disorders and the underlying mechanisms responsible.

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Chapter 5. Cognitive Bias and Depression-Like Behaviour

Abstract

Early-life adversity has been suggested to be involved in the etiology of mood disorders such as depression. Whilst this cannot readily be tested experimentally in humans, depression-like behaviour following exposure to early-life adversity can be measured in animal models using a cognitive approach. Pessimistic responses to ambiguous stimuli can be used as a measure of depression-like behaviour. In a cohort of hand-reared European starlings (*Sturnus vulgaris*), a two-by-two factorial design was used to simulate different levels of early-life adversity by separately manipulating food availability (Amount – Plenty vs Lean) and begging effort (Effort – Easy vs Hard) in the first two weeks post-hatch. We then measured cognitive bias in adulthood. Birds were trained to associate a coloured lid – positive – with a high-valued reward (a mealworm) and another coloured lid - negative – with nothing. After this discrimination had been successfully learned, subjects were tested by presentations of ambiguous stimuli intermediate between the two learned stimuli. Animals that responded to ambiguous stimuli similar to positive trials were interpreted as being highly expectant of reward, and birds that responded to ambiguous stimuli similar to negative trials were interpreted as displaying low expectation of reward and hence were considered more pessimistic. We found a significant effect of Effort on the latency to approach the first presentation of an ambiguous stimulus. Hard birds (those who had experienced increased begging effort during development) were significantly faster to respond to novel ambiguous stimuli and showed more optimistic-like behaviour. Interestingly, this effect was not strong enough to be detected when all ambiguous trials were pooled together over the 4 day testing period, potentially due to birds learning that intermediate trials were not rewarded. Finally we found that developmental treatments and HPA negative feedback affected learning capabilities. Plenty-Easy birds and birds with less efficient HPA axes (as indicated by higher ΔCORT in the CORT assay – see chapter 3) were faster to approach the learned negative stimulus during testing. Our results add to the literature that early-life adversity can alter mood and affective state.

5.1 Introduction

In humans, exposure to early-life adversity has been repeatedly linked to the development of affective disorders such as depression (including major depressive disorder and bipolar disorder) (Heim et al. 2008; Sadowski et al. 1999; Parker et al. 1995; Kendler et al. 2002). Depression is often characterised by symptoms such as social withdrawal fatigue, loss of pleasure (anhedonia) and reduced cognitive abilities, as well as being associated with hyperactivity of the hypothalamic-pituitary-adrenal system (DSM-IV 1994; Rüedi-Bettschen et al. 2005). This disease has a high economic and social cost, and yet the sources of individual differences in vulnerability to developing depressive disorders are not yet completely understood. Risk factors that are known to increase susceptibility of contracting mood disorders have been identified as both genetic and environmental, with particular interest placed upon exposure to adverse conditions. In 1999, a study conducted in Newcastle upon Tyne (UK) found that 28% of individuals exposed to measures of social adversity (such as divorce, illness, unemployment and overcrowded living conditions) in the first 5 years of life had suffered from depression by the age of 32, compared to just 7% of controls (Sadowski et al. 1999). Such findings have been replicated many times (Gilman et al. 2003; McLeod & Shanahan 1996; Weich et al. 2009), showing an increased risk of depression in those with high levels of social stress. Early nutritional stress has also been implicated as a risk factor, with babies of low birth weight shown to be at an increased risk of developing a mood disorder (Gale & Martyn 2004). Animal models provide a way to experimentally test the hypothesis that nutritional and social forms of early-life adversity can leave individuals susceptible to the development of depression.

Measuring depression-like behaviour with respect to early-life adversity in animals has been predominantly conducted in rodents, measuring relatively simple characteristics such as anhedonia, open field behaviour, immobility (representative of behavioural despair) and sucrose preference (Overstreet 2012), with mixed results. Maternal separation manipulations in rats, whilst effective in increasing anxiety-like behaviour (Kalinichev et al. 2002; Caldji et al. 2000), do not always cause anhedonic or impaired coping behaviour, reflective of depression (Crnic et al. 1981; Shalev & Kafkafi 2002). Rüedi-Bettschen et al. (2005) found evidence of reduced motivation to obtain a reward and increased immobility in a forced swim test, albeit under very specific conditions (rats had been separated from both dam and littermates for 4 hours as pups and tested during the dark phase of their circadian cycle at 21^{o}C). Behavioural tests such as these offer valuable information into an animals' current state but there are problems with interpretation of their results (Paul et al. 2005). For example, they may be measuring arousal (how 'activated' the animal is) but not valence (whether

this activation is positive or negative). More recently there has been a shift away from behavioural tests of depression towards the use of a more cognitive approach.

Negative affective states, as seen in depression, have the ability to influence how an individual makes a decision in an ambiguous situation, also known as their cognitive bias (Winkielman et al. 2007). For example, depressive patients are more likely to interpret scrambled sentences ("winner born I am a loser") in a more negative manner ("I am a born loser") than positive ("I am a born winner"). Cognitive bias paradigms such as these have subsequently been developed for use in non-human animals (Bateson et al. 2015; Harding et al. 2004). In a typical test of cognitive bias, subjects are trained to associate a stimulus (positive) with a reward and another stimulus (negative) with a punishment or with nothing. After this discrimination has been learnt, subjects are tested by the presentation of ambiguous stimuli. In these trials, 'optimistic' animals (responding to the ambiguous stimuli similarly to positive stimuli) are said to be in a more positive affective state, and 'pessimistic' animals (responding similarly to negative stimuli) are in a negative affective state.

The first study to assess cognitive bias in animals was conducted by Harding et al. (2004). Rats were first trained to press a lever when exposed to a tone that signalled food, and to refrain from pressing the lever in response to another tone that signalled that the rats would experience white noise. The authors found that animals in unpredictable housing conditions tended to avoid pressing the lever when exposed to novel ambiguous tones, consistent with a more negative affective state and a reduced expectation of reward. Other such manipulations designed to cause negative affective states have resulted in the 'pessimistic' interpretation of ambiguous stimuli across many species (Bateson & Matheson 2007; Bateson et al. 2011; Burman et al. 2009). Conversely, positive affective states are associated with 'optimistic' responses (Brydges et al. 2011). Important for mood disorder research, animals diagnosed with separation anxiety (in dogs) and congenital helplessness (in rats) also show negative bias in cognitive bias assessments (Enkel et al. 2010; Mendl et al. 2010). The usefulness of cognitive approaches to test for depression-like behaviour is clear, however the use of such studies with respect to early-life adversity has not been fully explored.

In 2012, Brydges et al. (2012) showed that early-life adversity can affect cognitive bias in an animal model. Here, rats were stressed as juveniles through exposure to a combination of restraint, forced swim and electric footshock stressors. Surprisingly, the authors found that animals from the stressed groups responded to ambiguous stimuli (measured here as the expectation of a food reward) in a more 'optimistic' manner than the control animals. Further studies have shown contrasting results, with stress in the form of isolation, overcrowding and cage tilting experienced in adolescence leading to adult rats exhibiting a negative cognitive bias (Chaby et al. 2013). To date, there is only one study

exploring the effect of early-life adversity on cognitive bias in birds. Bateson et al. (2015) trained birds to associate a coloured lid with the presence of a palatable mealworm (a reward), and a different coloured lid with the presence of a toxic quinine-injected mealworm. Bateson et al. (2015) demonstrated that birds that had been competitively disadvantaged as nestlings showed longer latencies to remove ambiguous lids, indicative of a negative cognitive bias. Although significant differences in latencies to approach the trained stimuli were detected, birds continued to consume the quinine-injected mealworms, making it difficult to interpret how the positive and negative stimuli (and therefore ambiguous trials) were regarded by each treatment group. It is worth mentioning that although quinine is an aversive substance, in this experiment, the worm is best thought of as a poorer reward as opposed to a punisher. Competitive disadvantage brood manipulations used in the experiment by Bateson et al. also make it difficult to tease apart the individual effects of physical nutritional stress and begging effort. Finally, this study did not take into account individual differences of how the brood manipulation affected key physiological systems known to impact upon the development of depression.

The relationship between mood disorders such as depression and early-life adversity is thought to be controlled by persistent changes in the HPA axis and circulating glucocorticoid concentrations (Holsboer 2000; Nemeroff and Vale 2005; Warnick et al. 2009). Decreased sensitivity to glucocorticoid hormones (corticosterone (CORT) found in birds, reptiles and amphibians and cortisol in most mammals and fish) is thought to promote depression-like behaviour by impairing the negative feedback system of the HPA axis (Holsboer 2000). Often, studies of depression account for differences in circulating CORT concentrations (for example, by timing experiments that control for daily rhythms in CORT production - Chaby et al. (2013)). However, measured CORT concentrations have not been incorporated into a study of cognitive bias as yet. Previously in this cohort, we have shown that the HPA axis can be influenced by early-life adversity with changes seen to both peak CORT concentrations in response to an acute stressor and the negative feedback system (as assayed by ΔCORT, the change in CORT levels between 15 and 30 minutes) (Chapter 3, Experiment 1). Combining this physiological data with a cognitive study of depression would add to the literature concerning early-life adversity, the development of mood disorders and the potential mechanisms that mediate this.

This study aims to further investigate the relationship between early-life adversity and the etiology of depressive phenotypes in a long-lived passerine species, the European starling (*Sturnus vulgaris*). In a cohort of hand-reared birds, a two-by-two factorial design was used to simulate different levels of early-life adversity by separately manipulating food availability and begging profitability in the first two weeks post-hatch (Nettle et al. 2017; Chapter 2). At the end of the manipulation, birds were raised in uniform conditions until the time of the adult cognitive bias experiments. Developmental

telomere attrition (DTA), a biomarker of biological age, was measured in erythrocytes and was found to be accelerated in birds who had experienced the lowest amount of food and the highest begging effort (Nettle et al. 2017; Chapter 2). Additionally, CORT profiles were measured between one and four months prior to behavioural testing (measuring baseline CORT concentrations, peak CORT concentrations post-exposure to an acute stressor and ΔCORT – a measure of negative feedback). Here we found a faster return to baseline CORT concentrations post-exposure to a stressor (increased ΔCORT) in birds that had experienced higher begging effort as chicks. We also showed reduced levels of peak CORT in birds that had experienced higher begging effort, but no restriction on the amount of food given (Chapter 3, Experiment 1).

Here, we report on the effects of developmental experience on affective state and subsequent depressive-like phenotypes using a cognitive bias task adapted from Bateson & Matheson (2007). Briefly, animals from the 2014 cohort were trained to associate a stimulus – positive – with a high-valued reward and another stimulus – negative – with nothing. After this discrimination had been successfully learned, subjects were tested by presentations of ambiguous stimuli intermediate between the two learned stimuli. Animals that responded to ambiguous stimuli in a similar manner to positive trials were interpreted as being highly expectant of reward, and birds that responded to ambiguous stimuli similar to negative trials were interpreted as displaying low expectation of reward and hence adopted a 'pessimistic' cognitive style, (indicating a negative affective state). We chose not to use a punisher in this experiment for several reasons. First, our sample size was limited (32 individuals) and we were relying on the birds to voluntarily engage with the experiment and did not want to deter them from participating. Second, the use of a punisher can make it difficult to assess if biases are due to an increased expectation of reward or a decreased expectation of punishment. Finally, practical and appropriate punishers for starlings would usually involve the use of unpalatable food. Several studies have previously shown that early-life adversity can alter how birds perceive such food and therefore there may be treatment differences with regards to how birds value the punisher (Bloxham et al. 2014; Bateson et al. 2015).

We hypothesised that the developmental treatments would alter expectation of reward, with birds from more adverse treatment groups having a more pessimistic-like response to ambiguous stimuli. We also predicted that shorter telomeres (greater DTA) would be associated with a poorer somatic state and therefore would also show more depressive-like behaviour than birds with less DTA. Finally, we predicted that increased basal and peak CORT concentrations and lower ΔCORT (indicative of impaired negative feedback) would be associated with reduced expectation of reward.

5.2 Materials and Methods

5.2.1 Housing and Husbandry

Subjects in this study were adult European starlings that had been subjected to a unique developmental manipulation described elsewhere (Chapter 2; Nettle et al. 2017), designed to dissociate the effects of overall quantity of food received (Amount – Plenty or Lean) from the begging investment required to obtain it (Effort – Easy or Hard). The experiment consisted of 32 birds aged between 5 and 9 months. (16M, 16F; 8LH, 8LE, 8PH, 8PE).

The cognitive bias experiment was conducted between October 2014 and February 2015, beginning when the birds were at a mean of 217 days post-hatch. Four replicates of eight birds (with two complete genetic families per replicate) were caught from indoor aviary housing and moved to the experimental room (18^{o}C, 35% humidity, 14:10 light cycle). Birds were individually caged (75x45x45cm) with access to two perches, a water bath and two *ad libitum* drinkers for the entirety of the experiment. *Ad libitum* food (cat food, domestic chick crumb (Special Diets Services 'Poultry Starter (HPS)') supplemented with fruit) and water baths were present at all times except during experimental sessions which ran for approximately two hours every morning. Mealworms were provided as rewards during these periods. Food was removed from cages one hour before trials were due to begin to ensure that birds were motivated to take part in the experiment. Birds remained in cages for between 2-3 weeks depending on the time taken for each replicate to learn how to flip lids. Birds were able to see and hear others at all times except during the experiment, in which birds were visually isolated using curtains. When a replicate had completed the experiment, the birds were returned to the aviary and replaced with the next two families. Birds were weighed upon entry to and exit from the experiment.

5.2.2 Cognitive Bias Assessment

5.2.2a Overview

In total, there were four stages of the experiment, detailed below (Figure 5.1). Briefly, cognitive biases were assessed using an adapted Go/No-Go task first used by (Bateson & Matheson 2007). Birds were initially trained to remove a lid covering a Petri dish glued to a plastic white tile containing an obscured mealworm within a 60 second time period. Discriminative stimuli in the form of different coloured lids were then introduced, indicating the presence (POS) or absence (NEG) of the worm. The colours of lids were achromatic percentages of grey scale (20% or 60% printed in black on white laminated card).

Each replicate was assigned either 20% or 60% as the POS and NEG stimuli respectively, counterbalanced across replicates. Birds were required to demonstrate successful learning of the difference between POS and NEG lids in the form of significantly faster latencies to remove the POS lids when compared to the NEG lids. There then followed a phase where the worm was only present on 75% of POS trials in which discrimination had to be maintained. To assess cognitive biases, birds were presented with both trained stimuli (POS and NEG lids) and ambiguous stimuli, intermediate to the 2 discriminating lids (NEARPOS, MID, NEARNEG lids - 30%, 40% and 50% grey scale tested in extinction). The latency to approach each ambiguous dish was used as a measure of cognitive bias. During all stages, the experimenter remained within the room, hidden from view and all trials were recorded for later scoring and analysis.

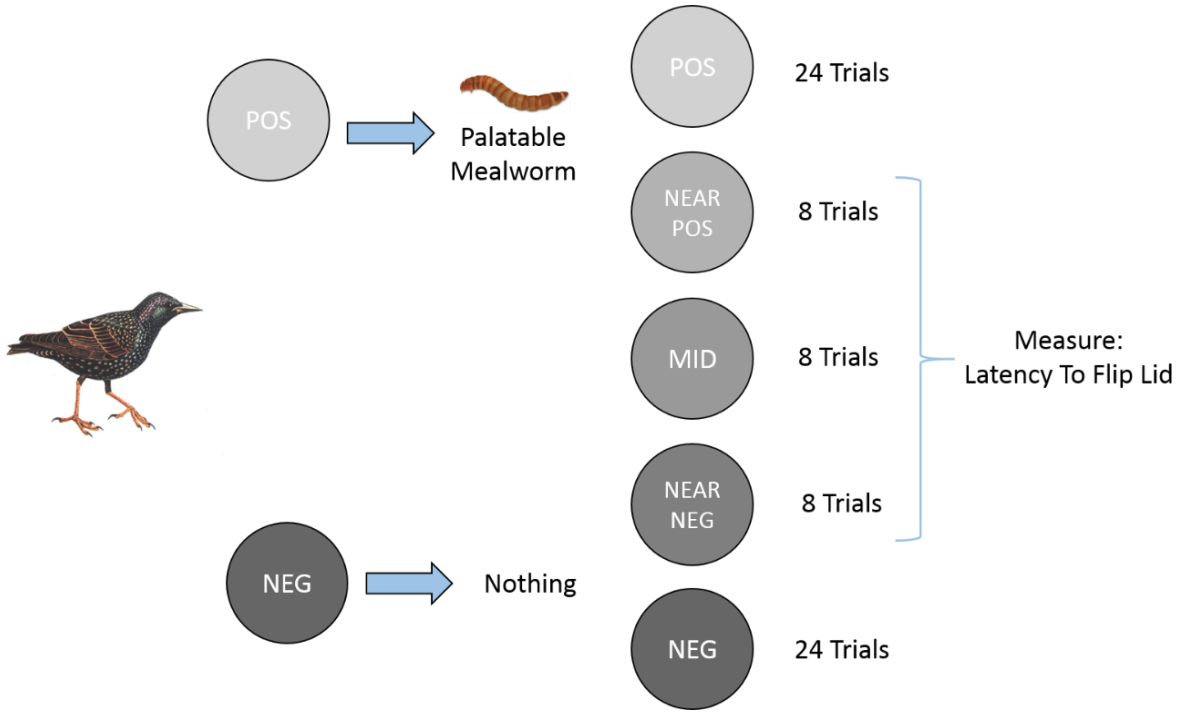

Figure 5.1: An overview of the experimental paradigm designed to test cognitive bias. First, birds were trained to associate a coloured lid with dishes that contained mealworms and a different coloured lid with dishes that contained nothing. Birds were then tested with 24 POS, 24 NEG, 8 NEARPOS, 8 MID and 8 NEARNEG trials with latency and propensity to remove an ambiguous coloured lid measured as a test of optimistic-like behaviour and expectation of reward.

5.2.2b Lid-Flipping Training

The aim of this stage was to train starlings to approach and remove a laminated cardboard lid covering a mealworm in a Petri dish within 60 seconds of the dish being presented. Birds were initially presented with two uncovered mealworms with adjacent assigned POS lids (either 20% or 60% grey scale) and given 10 minutes to eat the worm. In subsequent training, birds progressed through several stages involving the lid partially covering more of one mealworm until the bird was successfully able to flip fully covered dishes within 10 minutes. The presentation time of the dish was then reduced to

60 seconds. To move on to the next stage, all birds in the replicate had to successfully flip six out of eight presented lids within 60 seconds in any given set of trials. Birds were given a maximum of eight 10 minute trials, or 16 1 minute trials per day. The inter-trial interval (ITI) was approximately four minutes. One bird (Plenty-Hard treatment) was excluded after 30 unsuccessful training trials leaving 31 birds to complete the experiment.

5.2.2c Discrimination Training

The aim of this stage was to train the birds that one shade of grey is associated with a dish containing a mealworm, and another shade is associated with an empty dish. This training ensured that birds were faster, or more likely, to investigate POS lids than NEG lids. Birds were given 16 trials per day consisting of eight POS and eight NEG trials in a pseudorandomised order with a four minute ITI. All trials included a fully covered dish containing either a mealworm (POS) or nothing (NEG) and dishes were presented for 60 seconds. Constraints to presentation order included that all trials started with a POS trial and no more than two consecutive trials of the same type were presented. Successful discrimination was tested daily by comparing latencies to flip POS and NEG lids using non-parametric Mann-Whitney U tests. To progress to the next stage, birds had to have shown successful discrimination by responding significantly faster to POS lids than NEG lids on 2 consecutive days of trials.

5.2.2d Partial Reinforcement Training

The aim of this stage was to train the birds that not all POS trials were reinforced, to slow down extinction of flipping in the final testing stage. Birds received 16 trials per day, again consisting of eight POS and eight NEG trials in a pseudorandomised order with a four minute ITI. In this stage, two of the POS trials were not reinforced with the presence of a mealworm. Constraints included that all trials started with POS lids and no more than two consecutive trials of the same type were presented. Birds had to maintain successful discrimination on two consecutive days to pass on to the final stage of the experiment, again determined by Mann-Whitney U tests.

5.2.2e Cognitive Bias Testing

The aim of this phase was to measure the birds' responses to ambiguous stimuli, giving an indication of the animals' expectation of reward and its underlying affective state. The ambiguous stimuli were shades of grey intermediate to the two trained stimuli (POS and NEG). Three ambiguous stimuli were used; NEARPOS, MID and NEARNEG. All birds were subject to four consecutive days of 18 trials each consisting of six POS trials reinforced with a mealworm, and two NEARPOS, two MID, two NEARNEG

and six NEG non-rewarded trials given in a pseudorandomised order. The ITI was 4 minutes. Constraints included that all trials started with POS lids and no more than two consecutive trials of the same type were presented. Successful discrimination was checked at the end of this stage by comparing the latency to touch the POS and NEG lids combined over the four testing days using non-parametric tests. All 31 birds maintained successful discrimination throughout cognitive bias testing and all data was subsequently used in analyses.

5.2.3 Data Collection

Behaviour was recorded on two video cameras, each capturing four cages containing a family of birds. Birds were identifiable by coloured rings, however the experimenter remained blind to the treatment group to which they belonged. During all trials, an experimenter remained within the room, hidden from sight. Each trial was timed with a stopwatch and could be viewed live on monitors. Latencies to touch the lids with the beak and to subsequently eat the mealworms were scored by one experimenter after each day of the experiment from video recordings by hand. Trials started when the experimenter's hand had fully left the cage. If the bird did not touch the lid or the worm within the 60 second time period, then the latencies were recorded as the maximum trial time + 1 (in stage 2-4, for example, this was 61s).

5.2.4 Statistical Analysis

The dependent variables identified of interest in the first three stages of the experiment were the number of trials taken to complete lid-flipping training, the number of days taken to complete discrimination training and the number of days taken to complete partial reinforcement training. The variables tested in the final testing stage of the experiment were latencies to approach the learned stimuli (all POS and NEG), the latencies to approach the ambiguous stimuli (all NEARPOS, MID and NEARNEG) and the latency to approach the first presentation of the ambiguous stimuli (first NEARPOS, MID and NEARNEG). All data were log+1 transformed to improve model fit.

The effect of potentially confounding variables that were not balanced between treatment groups (body condition and sex) were assessed by testing each as a sole predictor on each bias variable. There were no effects of sex on any bias variable, however body condition significantly predicted latencies to approach learned stimuli and latencies to approach ambiguous stimuli. Body condition was therefore used as a covariate when analysing these variables. If body condition was not a significant predictor in the initial model, it was not retained for further analyses. Where analysing the effect of developmental treatments, the base model for each bias variable had fixed effects of Amount, Effort as well as the interaction between the two treatments. Where analysing the effect of DTA, the base

model included a fixed effect of DTA between day 5 and day 56 post-hatch. Where analysing the effect of CORT variables (measured in Chapter 3: Experiment 1), fixed effects included baseline CORT, peak CORT and ΔCORT. Valence (POS, NEARPOS, MID, NEARNEG, NEG – the overall 'attractiveness/aversiveness' of the stimulus) was used as a linear covariate in models testing learned latencies, ambiguous latencies and first ambiguous latencies, along with its interaction with Amount, Effort, DTA and CORT profile variables where appropriate.

Latencies to flip lids were limited to between 0 to 61 seconds, however censoring adjustments were not made in this data set as birds responded to ambiguous trials within the 60 second trial period in 62% of cases. When analysing cognitive bias, models were created first from the trained POS and NEG trials to establish whether developmental treatment had an effect on overall speed of probing in the cohort of birds. The mean speed of the bird was then calculated (the average latency of each bird to remove POS and NEG trials during testing) and used as a covariate in the analyses of ambiguous latencies and first ambiguous latencies to account for individual variation in probing speed.

5.3 Results

5.3.1 Sex and Body Condition

Average mass and body condition were significantly correlated (r = 0.98, *P* < **0.01***). Males had a significantly higher body condition than females (mean males 6.71, mean females -1.18, Table 5.1). There was a significant effect of Amount, but not Effort on body condition (mean LE 3.86, mean LH -0.49, mean PE 7.28, mean PH 0.27 , Table 5.1, Model 2).

Table 5.1: Output from linear mixed effect models predicting body condition from sex and experimental treatments. A * represents an interaction term.

Model Number	AICc	Dependent Variable	Fixed Predictors	LRT	*P*	N
1	200.8	Body Condition	Amount*Effort	2.32	0.13	31
			Sex	15.54	**< 0.01***	
2	199.7	Body Condition	**Amount**	8.45	**< 0.01***	31
			Effort	0.26	0.61	
			Sex	13.56	**< 0.01***	

5.3.2 Training Stages

5.3.2a Lid-Flipping Training

Birds took an average of 10.8 ± 4.9 (mean ± SD) trials to complete the lid-flipping training section of the experiment, defined by removing a presented lid covering a mealworm within 60 seconds of the trial commencing. We fitted a model with number of trials to learn to lid-flip as the dependent variable and this was not significantly affected by developmental treatments, DTA nor CORT profile (Table 5.2).

Table 5.2: Output from linear mixed effect models predicting flipping training time from experimental treatments, DTA and CORT variables. NB: Training time variable is log transformed to improve model fit. A * represents an interaction term.

Model Number	AICc	Bias Variable	Fixed Predictors	LRT	*P*	B (± SE)	N
3	40.5	Flipping Training	Amount*Effort	1.19	0.28	0.29 (0.28)	31
4	38.5	Flipping Training	Amount	0.04	0.84	-0.03 (0.14)	31
			Effort	0.93	0.33	-0.13 (0.14)	
5	30.1	Flipping Training	DTA	0.41	0.52	-0.19 (0.30)	27
6	39.0	Flipping Training	Cort0	1.90	0.17	0.03 (0.020	31
			Peak	0.97	0.32	< 0.01 (<0.01)	
			Delta	0.10	0.75	< 0.01 (<0.01)	

5.3.2b Discrimination Training

All 31 birds learnt the association between the POS stimuli and the presence of a mealworm, and between the NEG stimuli and the dish containing nothing. Birds took an average of 3.84 ± 1.53 days (mean ± SD) (Mann-Whitney tests $P < \mathbf{0.05}$*) to successfully complete the discrimination training stage of the experiment, defined as flipping POS lids significantly slower than NEG lids over two consecutive days. At the time of successful discrimination (the final two days of discrimination training for each bird), birds continued to remove both POS and NEG stimuli with averages of 97.6% and 45.6% respectively. We fitted a model with the number of days taken to acquire discrimination of the lid colours as the dependent variable and developmental treatments, DTA or CORT variables as fixed predictors (Table 5.3). We found no effect of developmental treatments nor DTA, however ΔCORT significantly predicted how long it took birds to learn the discrimination between the lid colours, with

higher ΔCORT (and therefore more efficient negative feedback of the HPA axis) associated with faster associative learning (Table 5.3, Model 10; Figure 5.2).

Table 5.3: Output from linear mixed effect models predicting discrimination training time from experimental treatments, DTA and CORT variables. NB Discrimination training time is log transformed to improve model fit. A * represents an interaction term.

Model Number	AICc	Bias Variable	Fixed Predictors	LRT	*P*	B (± SE)	N
7	128.4	Discrimination Training Time	Amount*Effort	< 0.01	0.93	0.09 (1.15)	31
8	125.3	Discrimination Training Time	Amount	< 0.01	0.94	0.04 (0.57)	31
			Effort	0.38	0.54	0.33 (0.57)	
9	114.0	Discrimination Training Time	DTA	0.38	0.55	-0.75 (1.27)	27
10	121.9	Discrimination Training Time	Cort0	0.37	0.54	-0.05 (0.09)	31
			Peak	< 0.01	0.95	< 0.01 (0.04)	
			Delta	4.31	**0.04***	-0.09 (0.04)	

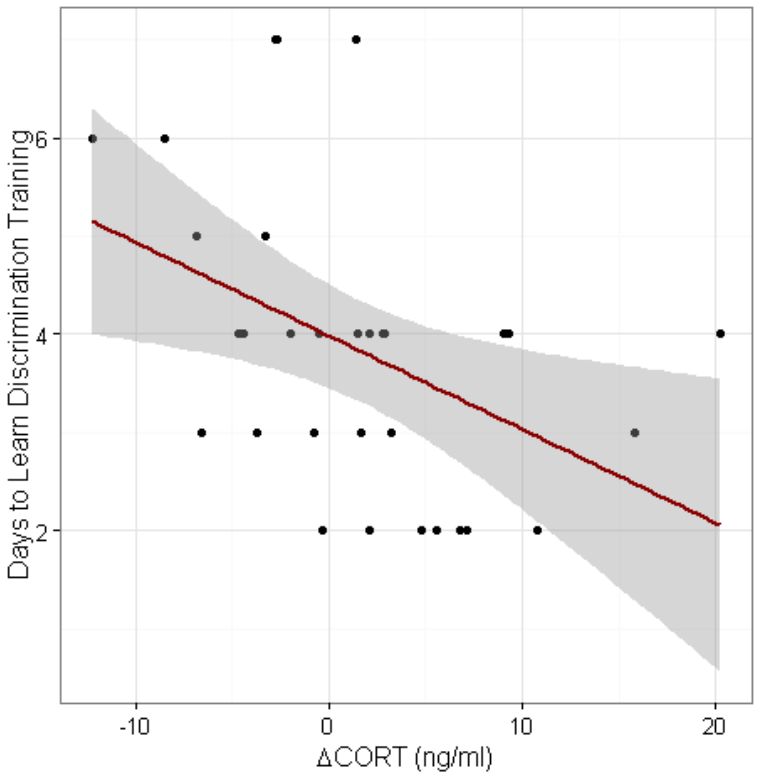

Figure 5.2: ΔCORT concentrations of 31 birds and how many days for each bird to acquire discrimination of the POS and NEG lids. Here, a more positive value of ΔCORT indicates a reduction in CORT concentrations between 15 minutes and 30 minutes post-stressor, and is representative of more efficient and faster negative feedback of the HPA axis.

5.3.2c Partial Reinforcement Training

All birds maintained discrimination between the POS and NEG stimuli into the partial reinforcement training stage, and completed this training in an average of 2.35 ± 0.80 (mean ± SD) days, demonstrated by continuing to flip POS lids significantly faster than NEG lids over two consecutive days (Mann-Whitney tests $P < 0.05$). At the time of successful discrimination with expected partial reinforcement (the final two days of reinforcement training), birds flipped an average of 96.7% POS lids and 39.3% NEG lids. There were no significant effects of developmental treatments, DTA nor CORT characteristics on the time taken to pass the partial reinforcement training stage (Table 5.4).

Table 5.4: Output from linear mixed effect models predicting days to pass partial reinforcement training from experimental treatments, DTA and CORT variables. NB Partial training time is log transformed to improve model fit. A * represents an interaction term.

Model Number	AICc	Bias Variable	Fixed Predictors	LRT	*P*	B (± SE)	N
11	18.2	Partial Training Time	Amount*Effort	1.79	0.18	-0.25 (0.19)	31
12	16.9	Partial Training Time	Amount Effort	0.41 0.24	0.52 0.63	-0.06 (0.10) - 0.05 (0.10)	31
13	6.9	Partial Training Time	DTA	0.60	0.44	-0.15 (0.20)	27
14	18.2	Partial Training Time	Cort0 Peak Delta	1.57 0.54 0.23	0.21 0.46 0.63	-0.02 (0.02) < 0.01 (<0.01) 0.45 (0.66)	31

5.3.3 Cognitive Bias Testing

5.3.3a Learned Stimuli

All birds maintained discrimination during bias testing (comparing the latencies of POS and NEG stimuli pooled from the 4 days of testing, Wilcoxon-ranked tests, all $P < 0.05$) and therefore all 31 birds were retained in the analysis of the cognitive bias testing phase of the experiment. Birds flipped 96.7% of POS lids and 25.1% of NEG lids (latencies to approach POS: 5.51 ± 11.39; NEG 48.6 ± 22.28 (mean ±SD)). We used a model fitting the latency to remove the learned stimuli with valence (POS or NEG), body

condition and either experimental treatments, DTA or CORT profile as the fixed predictors (Table 5.5). There was a significant three-way interaction of Amount, Effort and valence. Here, different treatment groups responded to POS and NEG lids at different speeds with the Plenty-Easy group being significantly faster to remove NEG lids (Table 5.5; Model 15, Figure 5.3). There was no significant effect of DTA on latency to remove learned lids. ΔCORT significantly predicted latency to remove the learned lids, with lower ΔCORT (and therefore less efficient cessation of the stress response) associated with faster latencies to remove the NEG lids (Table 5.5, Model 19; Figure 5.4).

Table 5.5: Output from linear mixed effect models predicting latencies to remove learned lids (POS, NEG) from experimental treatments, DTA and CORT variables. NB Learned latencies are log transformed to improve model fit. A * represents an interaction term.

Model Number	AICc	Bias Variable	Fixed Predictors	LRT	*P*	B (± SE)	N
15	3563.1	Learned Latencies	**A*E*Valence**	5.82	**0.02***	0.39 (0.16)	1488
			Body Condition	7.38	**< 0.01***	-0.04 (0.01)	
16	3063.1	Learned Latencies	DTA*Valence	0.10	0.75	-0.06 (0.18)	1296
			Body Condition	4.25	**0.04***	-0.03 (0.01)	
17	3061.2	Learned Latencies	DTA	0.44	0.51	-0.21 (0.31)	1296
			Valence	1588.26	**< 0.01***	2.39 (0.04)	
			Body Condition	4.25	**0.04***	-0.03 (0.01)	
18	3586.2	Learned Latencies	Cort0*Valence	2.02	0.16	-0.02 (0.01)	1488
			Peak*Valence	0.53	0.47	< 0.01 (<0.01)	
			Delta*Valence	0.04	0.83	< 0.01 (<0.01)	
			Body Condition	2.59	0.11	-0.02 (0.01)	
19	3583.0	Learned Latencies	Cort0	0.02	0.88	< 0.01 (0.03)	1488
			Peak	2.70	0.10	-0.02 (0.01)	
			Delta	6.74	**< 0.01***	0.04 (0.01)	
			Valence	1725.15	**< 0.01***	-2.32 (0.04)	

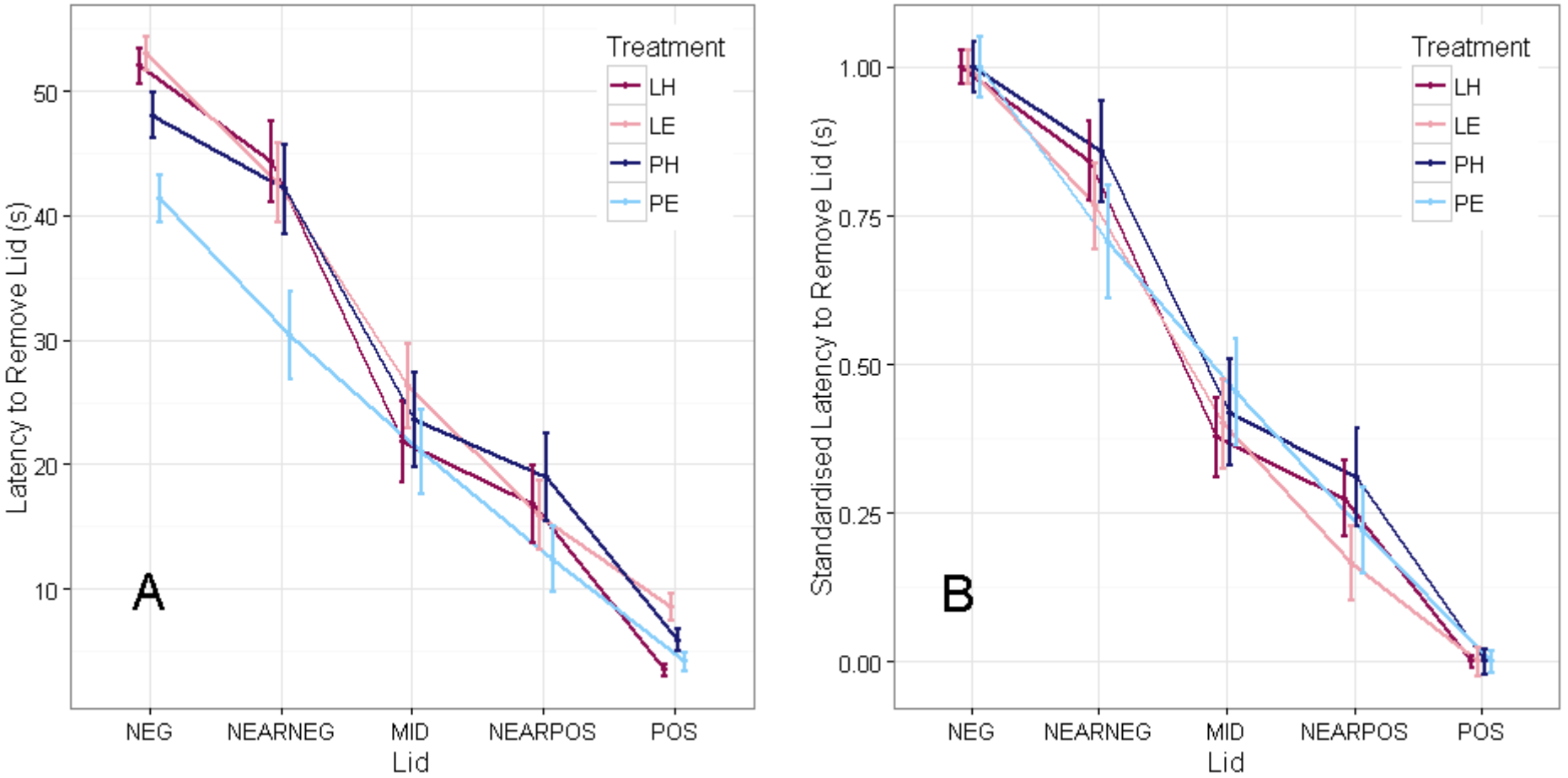

Figure 5.3: A - Latency to remove as a function of stimulus valence for birds in the four developmental treatment groups (LH = Lean-Hard, LE = Lean-Easy, PH = Plenty-Hard, PE = Plenty-Easy). Data shown are mean ± SE of the trial mean of latency to remove the lids in cognitive bias testing trials. B – The same data shown in panel A standardised so that the latencies to remove the learned POS and NEG stimuli are 0 and 1 respectively. This standardisation removes differences in mean speed to remove the learned stimuli seen between treatment groups and shows the differences in latencies to remove ambiguous stimuli when mean speed is controlled for (as seen in the analyses). Panel B is for visualisation purposes only.

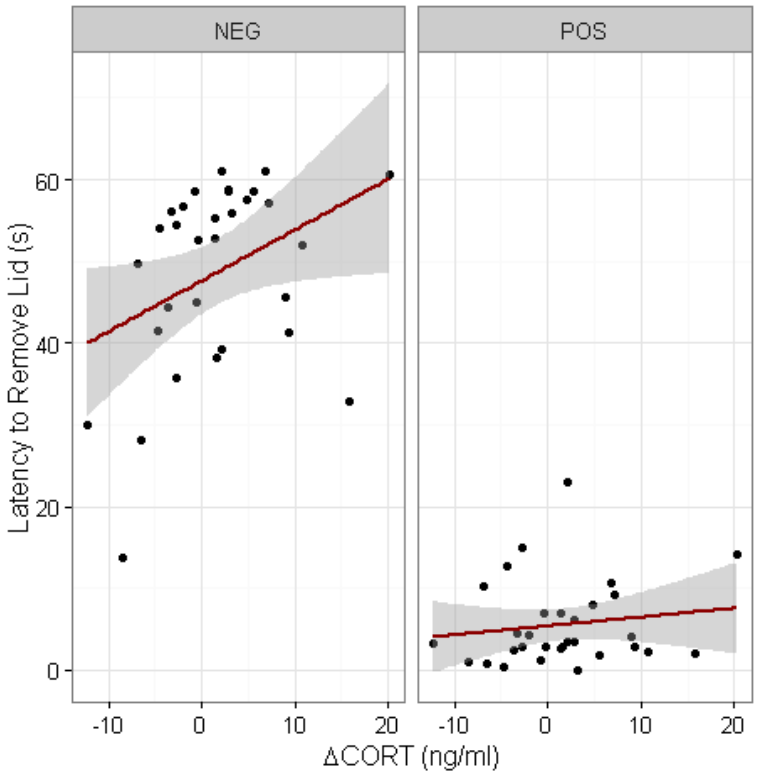

Figure 5.4: ΔCORT concentrations of 31 birds and latencies for birds to remove the learned stimuli (left panel – NEG, right panel – POS). Here, a positive value of ΔCORT indicates a reduction in CORT concentrations between 15 minutes and 30 minutes post-stressor, and is representative of more efficient and faster negative feedback of the HPA axis.

5.3.3b Ambiguous Stimuli

Due to treatment and CORT effects on the latency to remove trained stimuli, the mean speed of the bird (the average speed to remove POS and NEG stimuli for each bird) was calculated and used as a covariate in subsequent analyses of ambiguous stimuli latencies. The mean speed of the birds was 27.06 ± 7.31 (mean ± SD) seconds. In total, 79.0% of NEARPOS lids, 67.7% of MID lids, 38.7% of NEARNEG lids were removed. Average latencies to remove lids were NEARPOS 15.91 ± 23.55; MID 23.2 ± 26.84; NEARNEG: 39.8 ± 27.13 (mean ± SD). We fitted a model of latency to remove all tested ambiguous lids as the independent variable with mean speed (continuous), valence (NEARPOS, MID, NEARNEG) and either experimental treatments, DTA or CORT variables as fixed predictors (Table 5.6). There was a significant effect of valence throughout: There were no effects of developmental treatments (Table 5.6, Model 20-22; Figure 5.3), DTA (Model 23-24) nor CORT profile (Model 25-26) on latencies to remove all ambiguous stimuli.

Table 5.6: Output from linear mixed effect models predicting latencies to remove ambiguous lids (NEARPOS, MID, NEARNEG) from experimental treatments, DTA and CORT variables. NB Ambiguous latencies are log transformed to improve model fit. A * represents an interaction term.

Model Number	AICc	Bias Variable	Fixed Predictors	LRT	*P*	B (± SE)	N
20	2306.0	Ambiguous Latencies	A*E*Valence	0.09	0.95	0.12 (0.39)	744
			Mean Speed	27.93	**< 0.01***	0.08 (0.01)	
			Body Condition	1.11	0.29	-0.02 (0.01)	
21	2300.9	Ambiguous Latencies	Amount*Effort	0.14	0.71	-0.10 (0.28)	744
			Amount*Valence	1.02	0.60	0.10 (0.20)	
			Effort*Valence	3.13	0.21	0.23 (0.20)	
			Mean Speed	33.56	**< 0.01***	0.08 (0.01)	
22	2294.9	Ambiguous Latencies	Amount	0.21	0.65	0.06 (0.14)	744
			Effort	0.01	0.92	0.01 (0.13)	
			Valence	152.14	**< 0.01***	0.85 (0.10)	
			Mean Speed	34.43	**< 0.01***	0.08 (0.01)	
23	2026.5	Ambiguous Latencies	DTA*Valence	0.05	0.97	-0.10 (0.46)	648
			Mean Speed	23.72	**< 0.01***	0.09 (0.01)	
			Body Condition	1.34	0.25	-0.02 (0.02)	
24	2021.7	Ambiguous Latencies	DTA	0.06	0.81	0.08 (0.34)	648
			Mean Speed	24.89	**< 0.01***	0.09 (0.01)	
			Valence	133.13	**< 0.01***	0.87 (0.11)	
25	2303.4	Ambiguous Latencies	Cort0*Valence	1.13	0.57	0.03 (0.03)	744
			Peak*Valence	1.81	0.40	-0.01 (0.01)	
			Delta*Valence	1.05	0.59	-0.01 (0.02)	
			Mean Speed	27.38	**< 0.01***	0.07 (0.01)	
			Body Condition	1.68	0.19	-0.02 (0.01)	
26	2294.7	Ambiguous Latencies	Cort0	0.02	0.90	< 0.01 (0.03)	744
			Peak	0.08	0.78	< 0.01 (0.01)	
			Delta	1.21	0.27	0.01 (0.01)	
			Valence	152.13	**< 0.01***	0.85 (0.10)	
			Mean Speed	31.16	**< 0.01***	0.08 (0.01)	

We also isolated the latency to respond to the first presentation of NEARPOS, MID and NEARNEG stimuli to analyse the effect of developmental treatments, DTA and CORT profile on cognitive bias in an alternative manner (Table 5.7). There was no effect of DTA nor CORT profiles, however Effort significantly predicted the latency to approach the first presentation of an ambiguous stimulus. Here, Hard birds (those that experienced increased begging effort) were faster to approach ambiguous lids on the first presentation when compared to Easy birds (Table 5.7, Model 29; Figure 5.5).

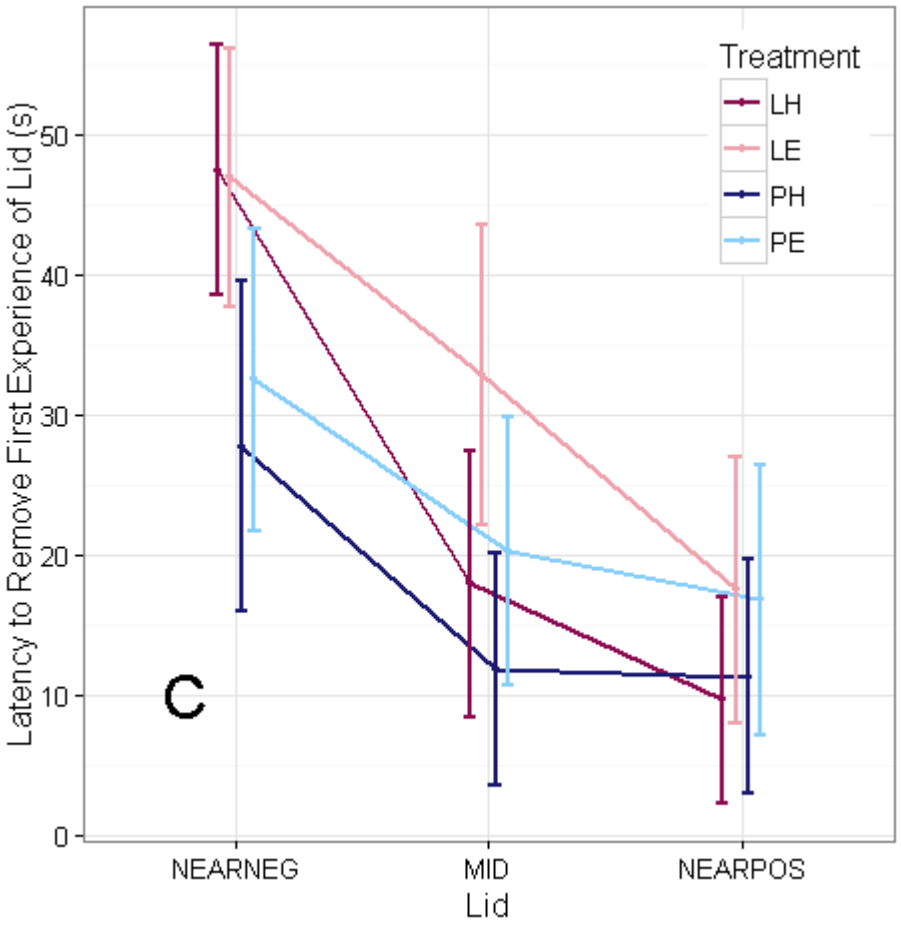

Figure 5.5: Latency to remove the first ambiguous stimulus presented as a function of stimulus valence for birds in the four developmental treatments (LH = Lean-Hard, LE = Lean-Easy, PH = Plenty-Hard, PE = Plenty-Easy). Data shown are mean ± SE of latency to remove the lids in cognitive bias testing trials.

Table 5.7: Output from linear mixed effect models predicting latencies to first remove ambiguous lids (NEARPOS, MID, NEARNEG) from experimental treatments, DTA and CORT variables. NB First ambiguous latencies are log transformed to improve model fit. A * represents an interaction term.

Model Number	AICc	Bias Variable	Fixed Predictors	LRT	*P*	B (± SE)	N
27	304.7	First Ambiguous Latencies	A*E*Valence	1.17	0.56	-0.99 (1.05)	93
			Mean Speed	27.55	**< 0.01***	0.19 (0.02)	
28	297.3	First Ambiguous Latencies	Amount*Effort	1.52	0.22	-0.52 (0.44)	93
			Amount*Valence	3.16	0.21	-0.31 (0.52)	
			Effort*Valence	0.85	0.65	0.44 (0.52)	
			Mean Speed	32.71	**< 0.01***	0.10 (0.02)	
29	289.9	First Ambiguous Latencies	Amount	0.03	0.87	-0.04 (0.23)	93
			Effort	4.03	**0.04***	-0.42 (0.21)	
			Valence	28.67	**< 0.01***	0.97 (0.26)	
			Mean Speed	30.30	**< 0.01***	0.10 (0.02)	
30	267.5	First Ambiguous Latencies	DTA*Valence	0.60	0.74	-0.75 (1.24)	81
			Mean Speed	15.85	**< 0.01***	0.11 (0.02)	
31	260.3	First Ambiguous Latencies	DTA	3.30	0.07~	1.01 (0.57)	81
			Mean Speed	15.84	**< 0.01***	0.11 (0.02)	
			Valence	23.53	**< 0.01***	0.91 (0.29)	
32	313.6	First Ambiguous Latencies	Cort0*Valence	0.64	0.73	0.02 (0.10)	93
			Peak*Valence	0.39	0.82	-0.02 (0.04)	
			Delta*Valence	0.63	0.73	0.02 (0.04)	
			Mean Speed	22.57	**< 0.01***	0.10 (0.02)	
33	295.6	First Ambiguous Latencies	Cort0	1.30	0.25	-0.05 (0.05)	93
			Peak	1.60	0.21	0.02 (0.02)	
			Delta	0.05	0.82	< 0.01 (0.02)	
			Valence	28.38	**< 0.01***	0.97 (0.26)	
			Mean Speed	24.38	**< 0.01***	0.10 (0.02)	

5.4 Discussion

5.4.1 Summary of Results

The aim of this study was to determine how differential experience of two naturally relevant forms of early-life adversity could affect depression-like mood in adults. We used a cognitive bias task designed to measure expectation of reward in the presence of ambiguous stimuli between stimuli previously associated with a food reward and with nothing. We predicted that birds from more adverse treatment groups and those with more DTA would be less likely to approach ambiguous stimuli, and would have greater latencies to remove these lids. We also predicted that birds with higher levels of circulating CORT (both baseline and peak) and with lower ΔCORT (indicating poorer negative feedback) would show more pessimistic-like behaviour in the presence of the ambiguous lids. Whilst we found no effect of treatments, DTA or CORT profile on ambiguous latency data averaged across all presentations, we did find a significant effect of Effort on the latency to approach the first presentation of an ambiguous stimulus. Hard birds (those who had experienced increased begging effort during development) were significantly faster to respond to novel ambiguous stimuli than Easy birds (those who had experienced minimal begging effort) and showed more optimistic-like behaviour.

We found both treatment effects and CORT associations with latencies to remove the learned stimuli during the testing phase of the experiment. We showed that Plenty-Easy birds (those from the least adverse developmental conditions) were faster to remove the learned negative stimulus during the testing phase. We also found an association of ΔCORT with how birds reacted to the negative stimulus, with lower ΔCORT (suggesting less efficient negative feedback of the HPA system) associated with faster latencies to remove the negative stimulus. Finally, we found a relationship of ΔCORT with associative learning speed, with birds with better negative feedback learning the discrimination between the positive and the negative lids in a faster time than birds with more negative ΔCORT.

5.4.2 Methodological Comments

We made several assumptions and experimental considerations for the design and analyses of this study. The Go/No-Go task used was first developed by Bateson & Matheson (2007) and assumes birds will learn the association between the positive stimuli and the reward, and between the negative stimuli and nothing. Whereas our data strongly support this assumption, the majority of birds continued to remove negative stimuli throughout all stages of the experiment albeit at a greatly reduced rate and with significantly slower latencies. This is to be expected as the negative stimuli used provided no deterrent and therefore behaviour towards this stimulus cannot be interpreted as an expectation of punishment. For reasons outlined earlier, our experiment was designed to assess

expectation of reward only, and by extension, depression-like mood, and cannot be used as a measure of anxiety-like behaviour (instead refer to chapter 4 for a separate experiment examining anxiety).

The analyses used in this experiment reflect those used by Bateson et al. (2015), in which the authors outline their rationale for analysing ambiguous stimuli latencies separately to the learned stimuli, as opposed to other recommendations (eg Gygax (2014)). In our experiment, learned and ambiguous stimuli were presented at different frequencies with different reward patterns to each other. These methodological differences argue against combining learned and ambiguous data when analysing latencies. By using ambiguous trial data independently of the learned trials, we were also able to reduce the number of censored trials (the number of trials in which birds exceeded the maximum trial length). Due to treatment differences with respect to the speed that birds approached trained stimuli in the testing trials, we used the mean speed of the learned trials for each bird as a covariate in the ambiguous stimuli analyses. Pragmatically speaking, this provides a less complex, but statistically robust method for interpreting our data when compared to the fitting of sigmoidal curves to latency data across all stimuli valences, as recommended by Gygax (2014).

Finally, there are limitations to evaluating the results from the ambiguous stimuli pooled together (8 NEARPOS, 8 MID and 8 NEARNEG presentations over the testing stage of the experiment). There is an argument that only the first exposure to the ambiguous stimulus can be truly reflective of an animal's cognitive bias and affective state, with subsequent exposure to the ambiguous stimuli being heavily influenced by previous unrewarded experience and individual learning capabilities. As we showed CORT-driven differences in associative learning, we also provided an analysis of latency data solely from the first presentation of the ambiguous stimuli (as seen in Brilot et al. (2010) and Doyle et al. (2011)) to accompany our pooled ambiguous stimuli analysis.

5.4.3 Ambiguous Stimuli and a Depression-Like State

We found a significant effect of Effort on latency to approach the first presentation of the ambiguous stimuli, with Hard birds being significantly faster than Easy birds to remove the lids. Contrary to our predictions, this is indicative of Hard birds perhaps having an increased expectation of reward and a resultant optimistic-like state. Interestingly, these birds were also shown to have increased ΔCORT and a faster return of CORT concentrations to baseline levels (Chapter 3). The literature regarding the effect of early-life adversity and cognitive bias provides mixed results with several studies demonstrating that juvenile stress leads to more pessimistic cognitive biases (Bateson et al. 2015; Chaby et al. 2013), however some studies find, similar to our experiment, the opposite effect (Brydges et al. 2012). Our experiment provides evidence that early-life adversity has the potential to cause long-lasting effects on cognitive bias and affective state, however in a more positive way than predicted.

There are several possible explanations for this difference in decision-making aside from intrinsic cognitive biases. First, Hard birds could be interpreted as more risk prone than Easy individuals. Previous studies have shown that developmentally stressed animals can increase levels of risk-taking behaviour when foraging (Killen et al. 2011; Damsgard & Dilp 1998). These studies were performed on animals that had undergone dietary restriction prior to the experiment and therefore may have been trying to compensate for reduced body weight. We showed in our experiment that there was a significant effect of Amount, but not Effort on body condition, indicating that there was no difference in body weight between Easy and Hard individuals. If Hard birds are indeed more risk-prone than Easy birds, their motivation for doing so is not due to a lower body condition. A second reason for the difference in Hard and Easy birds could be that developmental stress increases impulsive behaviour and subsequently decreases the length of time an animal takes to make a decision. Several studies have shown that developmentally stressed individuals demonstrate more impulsive behaviour by either reduced decision making time (Bateson et al. 2015; Brydges et al. 2012) or hyperactivity (Colorado et al. 2006) and indeed this is reflected in the human literature (Lovallo 2013). Finally, it is possible that Hard birds, whilst not being in a poorer body condition, show a demonstrably 'hungry' phenotype and are highly food motivated. Studies using European starlings have repeatedly shown that exposure to developmental stress (such as competitive disadvantage) led to hungrier adults who were also more likely to consume toxic prey than birds that had experienced a competitive advantage (Bateson et al. 2015; Bloxham et al. 2014). However, if Hard birds are indeed riskier, impulsive or show a more 'hungry' phenotype, it would be predicted that these birds would also have faster latencies to investigate learned stimuli, which does not appear to be true. Therefore, our original explanation that early-life adversity can alter cognitive bias and increase optimistic-like behaviour in starlings seems most likely. We propose that the use of tasks that do not depend solely on food rewards are crucial to determine how generalizable our results are with respect to cognitive bias.

Interestingly, when we analysed latencies to remove all presentations of the ambiguous stimuli pooled together, we found no effect of developmental treatments on affective state in this cohort of European starlings. This may be due to the birds learning that the ambiguous stimuli do not provide a reward over the four days of the testing stage. A previous cohort of European starlings showed a pessimistic cognitive bias across all tested ambiguous stimuli when exposed to developmental stress (Bateson et al. 2015), however there are several differences between this study and ours. First, the developmental manipulation used in Bateson et al. (2015) used brood size manipulations which exposed chicks to both physical (nutritional availability) and social (number of competitive siblings) stressors that cannot be analysed independently. Second, birds in the 2015 study were not hand-reared and therefore this study could not account for differences in parental quality, quantities of

food received or for the amount of begging birds were subjected to. Finally, cognitive bias in Bateson et al. was also measured in a different manner, beginning at a much younger age (mean 94 days post-hatch vs mean 217 days post-hatch in our experiment) and involved the use of a quinine-injected worm predicted by the negative stimulus.

We found no effect of DTA on latencies to remove ambiguous stimuli (either at the first presentation, or pooled over all testing trials). The human literature demonstrates that the relationship between telomere attrition and depression is complex, with separate studies finding either no effect of telomere length on major depression, or an association between shorter telomeres and pessimism (Needham et al. 2015; O'Donovan et al. 2009). In birds, Bateson et al. (2015) found that adults with greater levels of DTA showed shorter ambiguous latencies and were in a more positive affective state than birds with less DTA. It is clear that more studies into DTA and cognitive bias are needed across species to evaluate its contribution to the development of depression. We also found no effect of CORT profiles (neither baseline CORT, peak CORT nor ΔCORT) on cognitive bias. In humans however, lower levels of basal cortisol have been associated with higher levels of optimism and positive affective state (Lai et al. 2005; Endrighi et al. 2011). Importantly, Endrighi et al. (2011) did not find any effect of peak cortisol, nor ΔCORT on optimism. HPA function is strongly associated with the development of stress-related disorders, however we saw no effect of alterations to this axis on cognitive bias in our animals. A potential explanation is that the variation measured in our CORT samples is not big enough to have an impact on cognitive bias.

5.4.4 Learned Stimuli and Associative Learning

A three-way interaction between Amount, Effort and valence showed that Plenty-Easy birds were significantly faster to remove negative stimuli throughout the testing trials. Incidentally, Easy birds were previously shown to have reduced ΔCORT (Chapter 3, Experiment 1) and Plenty-Easy birds were in better body condition during this experiment. This was reflected in the testing data, with birds with lower ΔCORT and higher body condition also being faster to remove negative lids. It is possible that differences in ΔCORT provide a possible mechanism for which Plenty-Easy birds remove negative stimuli faster than other treatment groups (either through changes to associative learning systems, or through how aversive negative stimuli are perceived).

To provide support for the theory of associative learning mediating a difference in negative lids removed, we found a significant effect of ΔCORT on the speed of associative learning, namely the number of trials taken to learn the discrimination between the positive and negative stimuli. Birds with increased ΔCORT were faster to learn this distinction. Therefore we argue that reduced ΔCORT detected in the Plenty-Easy birds reduced their ability to learn and remember the difference between

the positive and negative stimuli, and thus led to these birds being more likely to investigate negative stimuli during testing than other treatment groups. We did not see this effect on the positive stimulus, potentially due to the presence of the worm being a more memorable association to the positive stimulus, than nothing was to the negative stimulus. Exposure to CORT has the potential to facilitate learning and memory processes (Oitzl & de Kloet 1992; Sandi & Rose 1994), however, prolonged exposure to CORT (caused by reduced efficiency of HPA negative feedback or through an increased latency to reach peak CORT concentrations) has been shown to negatively impact upon these systems, with retrieval of emotional memory particularly affected (Stegeren 2009). Here, we provide evidence to support the role of increased ΔCORT (faster return to baseline conditions and an earlier peak in CORT) and increased learning capabilities, however experiments that directly measure this association are needed.

5.4.5 Conclusions

Our experiment provides modest evidence to support the theory that early-life adversity can affect expectation of reward and affective state in a cohort of European starling. Animals that had experienced increased begging effort as juveniles were more likely to respond to ambiguous stimuli 'optimistically' at the first presentation, however this effect was not strong as it disappeared as ambiguous trials continued to be presented over a period of four days. We did find unexpected evidence that our developmental treatments were capable of altering how birds responded to learned stimuli, with animals that had experienced the least amount of developmental adversity being significantly quicker to remove negative stimuli than other treatment groups. We speculated that this may be due to differences in associative learning and memory capabilities. Indeed, this was corroborated by data collected from the HPA axis. Birds with lower levels of ΔCORT (shown to be Easy birds in Thesis Chapter 3, Experiment 1) were also faster to remove negative stimuli and took longer to learn the association between the positive and negative stimuli.

Our study contributes evidence to the theory that early-life adversity (in particular, begging effort) can alter affective state, however we did not find that our more adverse developmental treatments could cause depressive-like phenotypes. We showed that separate sources of adversity can impact upon mechanisms responsible for optimistic and pessimistic responses, implying that a direct link between adversity and the development of mood disorders such as depression is plausible. We did not find evidence to support the theory that depressive phenotypes could be mediated through alterations to the HPA axis, therefore other mechanisms must be considered. We can continue to investigate the effects of early-life adversity throughout the lives of these birds, taking into account further

physiological and behavioural measures to provide a comprehensive picture of the effects of environmental experience on the adult phenotype.

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Chapter 6. Global DNA Methylation

Abstract

Experience of early adversity has profound consequences for health and wellbeing, with DNA methylation being proposed as a mechanism to 'record' this environmental exposure. Methylation (the addition of a methyl group to DNA nucleotides) has many biological functions and consequences for gene expression and overall phenotype. Our aim was to measure the effect of early-life adversity on global DNA methylation in European starlings (*Sturnus vulgaris*) via two different methods. In this study, we asked how two natural forms of early-life adversity could affect global DNA methylation profiles, and if these results were long-lasting (by measuring methylation at 4 and 18 months of age). In a cohort of hand-reared European starlings (*Sturnus vulgaris*), a two-by-two factorial design was used to simulate different levels of early-life adversity by separately manipulating food availability (Amount – Plenty vs Lean) and begging effort (Effort – Easy vs Hard) in the first two weeks post-hatch. To measure DNA methylation, we used a modification of the comet assay, incorporating two restriction endonucleases (*Hpa*II and *Msp*I) that differ in sensitivity to DNA methylation. By comparing the degree of DNA digestion achieved by these two enzymes, we were able to measure overall global DNA methylation at CpG sites in erythrocytes. At 4 months of age, there were significant differences in global CpG methylation between our treatment groups, with the amount of food experienced as a nestling causing changes in methylation profiles as adults. However, effects of early-life adversity were no longer detectable a year later. We further used an immunoassay commercial kit (ELISA) to measure overall levels of methylation (not restricted to CpG sites) in the genome. The kit, whilst being more expensive and having higher throughput, did not detect differences between treatments. We argue that global methylation at CpG sites specifically can be altered through exposure to early-life adversity, that effects can resolve over time, and can be simply analysed using the modified comet assay. We go on to discuss why nutrition may produce differential DNA methylation profiles in adult birds. Finally, we discuss the use of global DNA methylation studies when compared to gene-specific methylation analyses.

6.1 Introduction

Exposure to early-life adversity is associated with poor adult health outcomes in humans and other animals, with epigenetic processes (changes in gene expression that cannot be explained by changes in DNA sequence) being proposed to play a role in the biological underpinning of early-life exposure to environmental stress. Cytosine bases can be converted to 5-methylcytosine (5-mC) within the vertebrate genome by the addition of a methyl (CH_3) group by DNA methyltransferase enzymes. This common and stable epigenetic process is termed DNA methylation. DNA methylation can alter gene expression through interference with DNA-binding proteins and by modification of chromatin and nucleosome structures (Kass et al. 1997). A large proportion of 5-mC is found at 5'-CCGG-3' dinucleotides, known as CpG sites. Overall, in mammalian genomes, 5-mC makes up approximately 1% of all bases, and is present at 70-80% of all CpG sites (Ehrlich et al. 1982).

Methylation is an important process throughout development and increased methylation in key exon enhancer or promoter regions is associated with downregulated gene expression or even complete gene silencing (Jones et al. 1995; Jaenisch & Bird 2003; Berger et al. 2009). Changes in DNA methylation can happen at any life stage, and studies have demonstrated the sensitivity of methylation to aspects of the environment (Baccarelli et al. 2009; Zeilinger et al. 2013). Monozygotic twins, for example, have been shown to diverge in their DNA methylation profiles as they age (Fraga et al. 2005). There is evidence that both global, and gene-specific methylation changes can occur in response to stress exposure (Mychasiuk et al. 2011; Weaver et al. 2004). Of interest to us is the effect of past environmental exposure on DNA methylation profiles in later life.

Early exposure to adversity has the ability to influence gene activity, and epigenetics could provide an explanation of how these events can leave long-lasting effects on physiology and behaviour, long after the initial experience. For example, adolescents whose mothers self-reported as highly stressed during their 1st year had significantly increased levels of CpG methylation in subsets of the genome than those not exposed to stress, identified in buccal epithelial cells (Essex et al. 2013). Whilst interesting insights into both global and gene-specific methylation can be gained from studying humans directly, many confounding factors exist that are known to affect DNA methylation profiles, including differences in underlying genetics, current dietary habits, undiagnosed illness and other uncontrolled environmental exposures. Zhang et al. (2011) demonstrated that genome-wide DNA methylation could be characterised based solely on dietary pattern, with diets containing a high intake of fruit and vegetables having less global DNA hypo-methylation compared to a diet consisting of high quantities of meat, dairy and grains. Using animal models, it is possible to reduce this type of variation.

Both acute and early-life events such as reduced maternal care, poor nutrition and early trauma have been shown to result in longstanding changes in DNA methylation patterns in animals (Weaver et al. 2004; Tobi et al. 2014; Suderman et al. 2014; Anier et al. 2014). Restraint stress in rats has been shown to immediately decrease global DNA methylation in 3 areas of the brain compared to non-stressed animals (Rodrigues et al. 2015). Using female rats that were selected for either low- or high-levels of licking and grooming behaviour, Weaver et al. (2004) demonstrated that significant differences in DNA methylation of the exon 1_F glucocorticoid receptor (GR) promoter were apparent in offspring within the first week postpartum. This corresponded with variations to the HPA axis and altered fearful behaviour later in life. Witzmann et al. (2012) showed that these differences in methylation were not as apparent when rats were instead exposed to a stress paradigm later on at 12 weeks of age, indicating that sensitivity is greater earlier in life.

Global DNA methylation has been fairly well described in mammalian species, including humans, rats and even baboons (Unterberger et al. 2009), however DNA methylation in birds has been neglected in comparison. Further to this, the effect of early-life adversity on global DNA methylation profiles in birds is, as yet, unknown. The first avian species in which global DNA methylation profiles were investigated across several tissue types was the chicken (Li et al. 2011). This found tissue methylation patterns to be largely similar to that of mammals using MeDIP (methylated DNA immunoprecipitation) technology. This technique is relatively time-consuming, expensive, and is not available to animals for which a genome sequence is currently unavailable. Further to this, Gryzinska et al. (2013) found that, again similar to mammals, global levels of CpG methylation were shown to be age-dependent, decreasing from 29.89% in blood of one day old chicks, to 18.56% in blood of 32 week old hens. Patterns of methylation appear to be highly conserved across vertebrates, and therefore conclusions made from avian models should not be dismissed when applying to humans.

Alternate sequence-independent methods of assessing global DNA methylation are available and are continually being developed. The single cell gel electrophoresis assay (known as the comet assay) has traditionally been used to assess DNA damage in the form of strand breaks and lesions. When exposed to an electrical field, small, damaged fragments of DNA migrate away from the cell nucleus through an agarose gel. The amount of damage to the cell can be quantified by the appearance of the resulting DNA 'head' (containing undamaged DNA) and 'tail' (containing damaged DNA) when viewed under a microscope. A modification of this assay incorporating isochizomer restriction enzymes to detect levels of global DNA methylation was suggested by (Wentzel et al. 2010) and verified using cultured HepG2 cells exposed to the de-methylating agent 5-azacytidine. To measure global DNA methylation, *Hpa*II and *Msp*I isochizomers are used in conjunction with the comet assay. These enzymes recognise the same DNA base sequence incorporating a CpG site (5'CCGG-3'), however differ in their sensitivity

to DNA methylation. *Hpa*II is inhibited if either cytosine in the recognition sequence is methylated, leaving the DNA intact. Conversely, *Msp*I is less sensitive to the presence of methylation and will digest the sequence if the internal cytosine is methylated, fragmenting the DNA. Methylation can then be measured by running the enzyme-treated DNA through an electrical field and assessing the amount of fragmented DNA in the tail. Differential sensitivity of *Hpa*II and *Msp*I has been exploited in other techniques for the detection of global DNA methylation including the Luminometric assay (LUMA) and the cytosine extension assay (CEA) (Karimi et al. 2006; Pogribny et al. 1999). Differential sensitivities of *Hpa*II and *Msp*I can be used to gain a measure of global DNA methylation at CpG sites if exposed to an entire genome. Wentzel et al. (2010) found that the use of *Hpa*II and *Msp*I in the modified comet assay produced results comparable to that of CEA.

A popular, but comparatively expensive, method of measuring global DNA methylation is the use of commercially available enzyme-linked immunoassay kits (ELISA). ELISA-based techniques can measure levels of methylation across the entire genome at all cytosine bases, unrestricted to CpG sites (as in the modified comet assay) with the use of specific methylation-binding antibodies (Mizugaki et al. 1996) relative to known standard concentrations. However, there are also common problems reported with both intra- and inter-assay variability of ELISA methods. The standard curve produced is subject to high levels of inter-assay variation due to differences between microplates and experimental conditions (Jones et al. 1995) and serial-dilution error (Racine-Poon et al. 1991). Cross-reactivity between antibodies has been reported in ELISA techniques, which could subsequently distort the reported levels of DNA methylation (Faaber et al. 1984). It is therefore imperative that new methods of global DNA methylation detection are developed and validated, with particular emphasis placed upon the reliability, expense and sensitivity of these assays.

The aim of our study had two parts. First, we aimed to validate the use of the comet assay as a reliable method of measuring global DNA methylation in European starling red blood cells (RBCs) from the 2014 cohort. RBCs are nucleated in avian species, and are therefore a practical and appropriate source of DNA. Second, we aimed to investigate the effect of early-life adversity on global DNA methylation profiles in adult European starlings (*Sturnus vulgaris*), and to identify the extent that these effects are stable over time. In a cohort of hand-reared birds, a two-by-two factorial design was used to simulate different levels of early-life adversity by separately manipulating food availability and begging profitability in the first two weeks post-hatch (Nettle et al. 2017, Chapter 2). Following the end of the manipulation, birds were raised in uniform conditions until the time of the methylation experiments. Developmental telomere attrition (DTA), a biomarker of biological age, was measured in erythrocytes and was found to be accelerated in birds who had experienced the lowest amount of food and the highest begging effort (Nettle et al. 2017, Chapter 2). At approximately 4 and 18 months of age, blood

samples were taken from each bird. RBCs were extracted and analysed using a modification of the comet assay with methylation-sensitive restriction enzymes at both time points. We also assessed global DNA methylation levels at 4 months of age using a methylated DNA immunoprecipitation 5-mC DNA enzyme-linked immunosorbent assay (ELISA) kit.

To meet our first aim, we repeated the comet assay on 6 samples taken at 4 months age a year later (technical replication) to assess repeatability of enzyme restriction. We predicted that this would show the comet assay to be a reliable method of measuring global DNA methylation, with no differences attributed to the timing of the comet assay. With respect to our second aim, we predicted starlings from more adverse developmental treatments and those with more DTA would show a significantly different global DNA methylation profile than birds from less stressful treatments at both CpG sites (measured using the comet assay) and across the entire genome (measured using the ELISA kit). We predicted that DNA methylation profiles would be stable and unchanging between the time of 4 months and 18 months, measured by comparing results from the two samples taken from the same animals. Finally, we predicted that we would see similar methylation profiles as calculated by the ELISA kit and the comet assay conducted on the 4 month old samples.

6.2 Materials and Methods

6.2.1 Note on Experiment 1, Experiment 2 and Experiment 3

This study consists of two comet assay experiments (Experiment 1 and Experiment 2) and an ELISA experiment (Experiment 3) with samples taken from the same individuals at two different time points. Blood samples for Experiment 1 and Experiment 3 were collected by CA when birds were 4 months old and analysed by AG. Samples for Experiment 2 were both collected and used by AG when the birds were 18 months old. In Experiment 1 and Experiment 2, the methodology used was identical unless otherwise specified.

6.2.2 Subjects and Blood Samples

Subjects in this study were adult European starlings that had been subjected to a unique developmental manipulation described elsewhere (Chapter 2; Nettle et al. 2017), designed to dissociate the effects of overall quantity of food received (Amount – Plenty or Lean) from the begging investment required to obtain it (Effort – Easy or Hard). Experiment 1 and 3 consisted of 32 birds aged 127-134 day (16M, 16F; 8LH, 8LE, 8PH, 8PE). Experiment 2 consisted of 30 of the same birds aged 584-601 days (15M, 15F; 7LH, 7LE, 8PH, 8PE – 2 birds had subsequently died between experiments). For obtaining blood samples, birds were transferred to individual cages (75x45x45 cm; 18^{o}C, 40% humidity,

15L:9D light cycle). All birds maintained full auditory and visual contact with other birds and had access to *ad lib* food and water. Birds were undisturbed for a minimum of three nights excluding daily husbandry (changing papers, drinking water, food and water baths) before blood sampling.

To obtain blood samples (full methodology described in chapter 2), 120µl blood samples were taken as quickly as possible after catching (within three minutes). Further 120µl blood samples were taken at intervals of 15 minutes and 30 minutes from catching for a separate experiment. RBCs collected from the first blood sample were counted using a haemocytometer. Briefly, RBCs were diluted 1:200 in Gower's solution (12.5g Na_2SO_4, 33.3ml Glacial acetic acid and 100ml distilled water) and further diluted 1:1 with trypan blue. 10µl of the RBC solution was applied directly to the haemocytometer and RBCs in 1µl were calculated. Samples were stored at -20^0C until use in either the comet assay or the ELISA experiment. Immediately before use in the comet assay, samples were diluted 1:100 with 10mM sterile PBS (Phosphate Buffered Saline).

6.2.3 Experiment 1 and Experiment 2 Methodology

6.2.3a DNA Methylation Profiling Using the Comet Assay

Experiment 1 and Experiment 2 refer to analyses of global DNA methylation profiles using a modified comet assay on RBC samples from two separate time points. For Experiment 1, this was when the birds were approximately 4 months and for Experiment 2, this was when the birds were approximately 18 months of age. The modification of the comet assay protocol was taken from Wentzel et al. (2010), with slight changes made to accommodate our samples. Briefly, the modified comet assay uses the difference in methylation sensitivity of two restriction endonucleases (*Hpa*II – methylation sensitive and *Msp*I – methylation insensitive) to measure global DNA methylation levels of individual cells. *Hpa*II is inhibited by methylation and does not digest methylated CpG sites. *Msp*I is not affected by methylation and cuts DNA at every encountered CpG site. By comparing the degree of digestion achieved by the two restriction enzymes, the overall level of CpG methylation can be measured. Throughout, the protocol was performed under dimmed lighting to reduce further DNA damage.

Approximately 20,000 RBCs from each blood sample (1 blood sample per bird for experiment 1 and experiment 2) were mixed with 100µl 0.5% low melting point agarose (LMPA) and applied to frosted glass slides, pre-coated with a layer of 1% agarose to create our comet samples. 100µl comet samples were added to individual slides in the arrangement demonstrated in Figure 6.1 (2 samples per slide, equating to 6 samples per bird). Cover slips were applied and the slides were left to set for 15 minutes at room temperature. After removal of the slips, slides were submerged in lysis solution (5M NaCl, 400mM EDTA with 100ml 10% DMSO and 1ml 1% Triton X-100 added immediately before use) for a

minimum of two hours at 4°C to prepare nucleoids. Slides were removed, dried, and samples were pre-soaked in 100µl enzyme reaction buffer (Tango buffer, Fermentas) for 10 minutes to ensure optimal conditions for enzyme digestion.

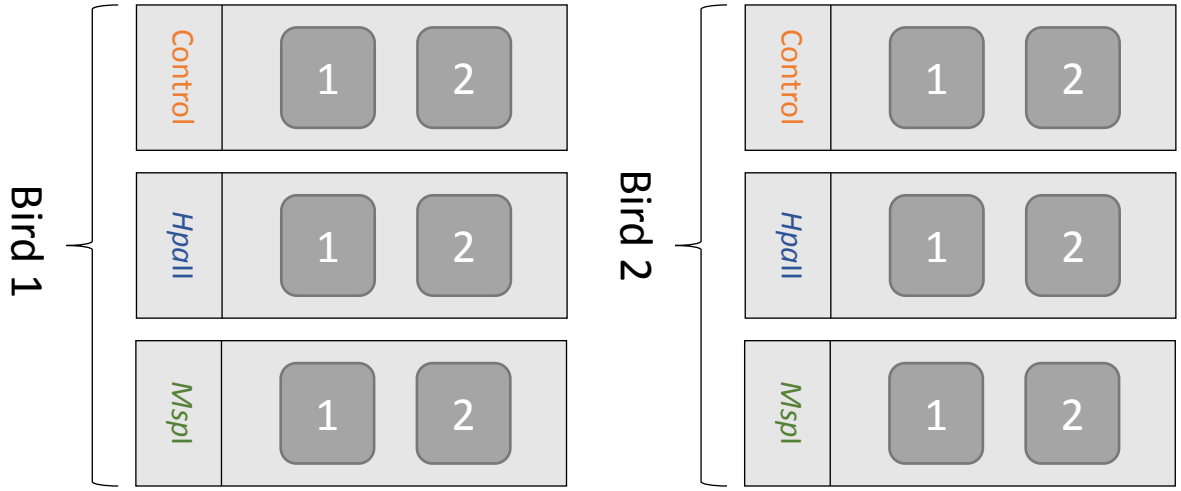

Figure 6.1: Layout of the slides used for two birds in the comet assay. 100µl samples were added to each slide in duplicate (1 and 2 on the diagram), with 3 slides allocated to each bird for control, *Hpa*II and *Msp*I treatments. Four birds were run on the assay simultaneously, grouped according to natal family.

Two separate enzyme mixtures were prepared containing either *Hpa*II or *Msp*I (Fermentas) (both mixtures 1.5U enzyme/100µl 1x Tango buffer) alongside a control mixture containing no enzyme (100µl 1x Tango buffer only). 100µl of each mixture was added separately to samples on each of the three slides and protected with a coverslip, followed by incubation in a pre-heated damp chamber at 37°C for one hour. Slips were removed and placed into a pre-chilled (4°C) electrophoresis tank and covered with electrophoresis buffer (5M NaOH, 400mM EDTA). Slides were rested for 30 minutes to allow DNA to unwind. Electrophoresis occurred at 30V and 300mA for 35 minutes. Subsequently, pH neutralisation was achieved by covering slides with a buffer (400mM Tris-HCl at pH 7.5) for 15 minutes to remove the alkali conditions and prime the slides for staining. Nucleoids were stained with 300µl diluted Sybr Gold (10µl in 10ml TBE, Life Technologies) and incubated for 45 minutes. Finally, slides were thoroughly rinsed with distilled water and left to dry overnight at room temperature in the dark.

Images of DNA heads and tails (known collectively as comets) were captured under UV light using a fluorescence microscope and analysed with Comet Assay IV software (Perceptive Instruments) within 48 hours. At least 50 comets were randomly scored per sample (with the exception of four samples that had fewer than 50 good quality comets), equating to over 300 comets scored per bird (minimum of 100 control, 100 *Hpa*II-treated and 100 *Msp*I-treated). Comets close to air bubbles and to the edges of the sample were avoided. Several different measures of comet formation are available through the Comet Assay IV software that are regularly used in the literature including tail intensity (the fraction

of total damaged or restricted DNA in the comet tail) and tail moment (a product of the tail length and tail intensity). Tail moment, a more robust measure, provides details about migration of the smallest detected strands as well as the number of broken DNA pieces in the tail of the comet. Both tail moment and tail intensity were highly correlated in all three enzyme treatments in experiment 1 (Control: R = 0.875, $P <$ **0.01*.** *Hpa*II: R = 0.885, $P <$ **0.01***, *Msp*I: R = 0.863, $P <$ **0.01***) and therefore tail moment was used as a measure of enzyme restriction throughout analyses of both experiments. Control comet tail moments (untreated starling RBCs) give information on overall DNA damage. *Hpa*II-treated tail moments were scored to give an indication of the amount of methylated CpG sites in the DNA sample. *Msp*I-treated tail moments indicated the total amount of CpG sites (regardless of methylation status) present in each DNA sequence. A slide containing two samples of cultured CaCo cells treated with methylating agent 5-azacytidine was used as a positive control. Positive controls were used in each run of the comet assay to confirm the success of the experiment, by way of a visual check. If the assay had been successful, the positive control comets consisted of very long DNA tails only.

6.2.4 Experiment 3 Methodology

6.2.4a DNA Extraction

DNA was extracted from RBCs taken when birds were 4 months of age using E.Z.N.A® Blood DNA Minikit (Omega Bio-Tek Inc, USA) in sibling batches with an adapted protocol as follows. 10µl of the RBC sample was added to 500µl 10mM sterile PBS in 1.5ml Eppendorf tubes in duplicate and vortexed at full speed for 60 seconds. Samples were rested at room temperature for 10 minutes, followed by vortexing again, and then placed in a heated shaking block at 65^{o}C for 10 minutes. 250µl of the sample was added to labelled 1.5ml Eppendorf tubes. 250µl BL buffer and 25µl OB Protease solution were added and vortexed for 15 seconds. After incubation at 65^{o}C for 10 minutes, 260µl 100% ethanol was added and the samples were vortexed for 20 seconds. Contents of the Eppendorf tube were transferred to HiBind® DNA Mini columns, placed in sterile nuclease-free 2ml microcentrifuge collection tubes. The manufacturer's protocol was herein adapted, with DNA eluted in 50µl elution buffer. 1.0µl DNA samples were quantified using NanoDrop ND1000 spectrophotometry initialised with H_2O and blanked with elution buffer, for indications of concentration and purity. DNA was stored at -20^{o}C until required. Samples recording less than 50ng/ml DNA were discarded.

6.2.4b Global DNA Methylation Profiling Using the ELISA Kit

Global DNA methylation analysis across the genome was performed using a commercially available MethylFlash™ Methylated DNA 5-mC Quantification Kit (Colorimetric) (Epigentek) based on the ELISA principle and used following the manufacturer's protocol. This kit was suitable for quantifying methylation from any species. Briefly, 150ng genomic DNA, negative control and positive control samples were added to wells in a 96-well microplate designed to have a high affinity for DNA and bound by incubation at 37°C for 90 minutes. Methylation levels were detected using diluted capture (specific to 5-mC) and detection antibodies which began the colorimetric reaction. Colour changes were visually monitored over 10 minutes. As per the manufacturer's instructions, reactions were stopped when colour in the positive control wells turned a medium blue. Positive controls in this assay were samples containing known levels of 50% 5-mC (0.5, 1.0, 2.0, 5.0 and 10.0ng/µl respectively). The negative control used was a polynucleotide consisting of 50% unmethylated cytosine. Quantification was through a microplate spectrophotometer at an absorbance level of 450nm. All bird samples were run in duplicate alongside the negative controls, positive controls and blanks (wells containing no DNA). Global DNA methylation levels were calculated as percentages with reference to a standard curve generated from the measured values from positive control samples. Regression of the standard curve was good (R^2 = 0.96). Average absorbance values for each bird were used for absolute quantification of DNA methylation using the formulae given below.

$$1) \quad 5-\mathrm{mC}\ (\mathrm{ng}) = \frac{\text{Sample Absorbance} - \text{Negative Control Absorbance}}{\text{Standard Slope} * 2}$$

$$2) \quad 5-\mathrm{mC}\ \% = \frac{5-\mathrm{m\ C}\ (\mathrm{ng})}{150}\ \mathrm{x}\ 100\%$$

There was very high intra-assay variation between the duplicates, with a coefficient of variation (CV) of 110.91%.

6.2.5 Statistical Analysis

The dependent variables used in experiment 1 and experiment 2 were: a) control tail moments (indicative of underlying DNA damage and b) *Hpa*II-treated tail moments (indicative of the amount of methylation present in each sample). For experiment 3, the dependent variable was percentage global DNA methylation as calculated by the kit. The effect of potentially confounding variables that were not balanced between treatment groups (body condition and sex) were assessed by testing each as a sole predictor on each dependent variable. There were no effects of sex or body condition on any

variable tested in the comet assay, nor the ELISA kit, and were therefore excluded from further analyses.

6.2.5a Experiment 1 and Experiment 2 Individual Analyses

We analysed tail moments for over 300 comets per bird in each experiment. In experiment 1, the minimum number of comets scored from 1 sample was 26 (*Msp*I-treated Plenty-Easy bird) due to a small number of cells present on the slide. One sample was recorded as 0 comets (*Msp*I-treated Plenty-Easy bird) due to loss of sample. In experiment 2, all samples contained at least 50 scoreable comets. To proceed with analysis of the comet assay results, validation criteria were set. We expected tail moments to increase across enzyme treatments, with control samples showing shortest tails (no restricted DNA in tails) and *Msp*I-treated samples showing the longest (large amount of fragmented DNA in tails). Our prediction was met in both experiments as we found that tail moments differed between our three assay treatments, with control samples having shorter tails than the two enzyme treatments. Negative control samples appeared circular (average tail moment experiment 1 (± SD): 8.89 ± 7.06, experiment 2 (± SD): 1.20 ± 0.94), while *Hpa*II-treated samples (average tail moment experiment 1 (± SD): 18.81 ± 12.74, experiment 2 (± SD): 15.52 ± 9.86) and *Msp*I-treated samples (average tail moment experiment 1 (± SD): 43.04 ± 13.49, 2 (± SD): 105.12 ± 28.22) had much longer tails, giving the nucleoids a more 'comet'-like appearance.

Wentzel et al. (2010) recommended the calculation of percentage global DNA methylation at CpG sites using a ratio of tail intensity in *Hpa*II- and *Msp*I-treated samples using the formula (100 - (*Hpa*II/*Msp*I)*100%). Instead of creating one summarising figure per bird, we used mixed effects models containing raw tail moment data as a dependent variable. The dependent variables explored in experiment 1 and experiment 2 were control and *Hpa*II-treated tail moments. Tail moment data were log+1 transformed to improve model fit. Where analysing *Hpa*II-treated samples, the mean of *Msp*I-treated tail moments was included as a covariate to control for underlying DNA damage and individual differences in DNA sequence. Where analysing the effect of developmental treatments, the base model of tail moment variables had fixed effects of Amount, Effort as well as the interaction between the two treatments. Where analysing the effect of DTA, the base model included a fixed effect of DTA between day 5 and day 56 post-hatch. Random effects of natal nest (n = 8), bird (n = 30-32) and sample (n = 2 per slide) were used throughout to control for non-independence of repeated measures.

To assess the repeatability of the comet assay, 6 samples used in experiment 1 were retested approximately a year later as technical replicates. Two-way ANOVAs were used on tail moment data from the three enzyme conditions with individual bird and time point (1 or 2) as fixed predictors.

6.2.5b Experiment 1 and Experiment 2 Combined Analyses

To compare the two experiments, it was necessary to combine the tail moment data from experiment 1 and 2 and retest the effect of confounding variables. In the combined dataset, body condition and sex did not affect control or *Hpa*II-treated tail moments. Mixed effects models using experiment (1 or 2) as a fixed predictor were tested on the combined control, *Hpa*II-treated and *Msp*I-treated sample tail moments. Random effects of natal nest and bird controlled for repeated measures.

6.2.5c Experiment 3 Analysis

The dependent variable explored in experiment 3 was % 5-mC methylation as calculated using the ELISA kit. When analysing the effect of developmental treatments, the base model had fixed effects of Amount, Effort and the interaction between treatments. Where analysing the effect of DTA, the base model had fixed effects of DTA between day 5 and day 56. Random effects of natal nest were used to control for repeated measures.

6.2.5d Comparing the Comet Assay and the ELISA

To compare results from the measures of global DNA methylation conducted on the same samples, from the first comet assay (experiment 1) and the ELISA kit (experiment 3), a model was fitted with % 5-mC methylation as the dependent variable and the mean of *Hpa*II-treated tail moments in experiment 1 as a fixed predictor. The mean of *Msp*I-treated tail moments in experiment 1 was included as a covariate in this analysis.

6.3 Results

6.3.1 Experiment 1

Experiment 1 refers to the comet assay conducted on blood samples taken from birds when they were approximately 4 months of age.

6.3.1a Control Tail Moments

To assess the effect of developmental treatments and DTA on underlying DNA damage, we looked at control tail moments. We ran a series of mixed effects models predicting control tail moments from developmental treatments (Amount, Effort) and from DTA (Table 6.1). There was no effect of treatment (Figure 6.2a) nor DTA on control tail moments, indicating no differences in basal DNA damage.

Table 6.1: Output from linear mixed effect models predicting control tail moments from experimental treatments and DTA in experiment 1. NB Control tail moment is log transformed to improve model fit. A * represents an interaction term.

Model Number	AICc	Dependent Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
1	10101.5	Control Tail Moment	Amount*Effort	0.08	0.78	-0.05 (0.18)	3276
2	10099.5	Control Tail Moment	Amount Effort	1.17 2.23	0.28 0.14	0.10 (0.10) 0.14 (0.10)	3276
3	8883.8	Control Tail Moment	DTA	0.27	0.60	0.14 (0.26)	2725

6.3.1b *Hpa*II Tail Moments

The effect of treatments and DTA on tail moments in *Hpa*II-treated samples were assessed using models with the mean of tail moments of *Msp*I-treated samples used as a covariate in analyses (Table 6.2). Amount significantly predicted tail moments of *Hpa*II-treated samples with Lean birds showing the shortest tail moments when treated with *Hpa*II (Table 6.2, Model 5; Figure 6.2b). Plenty birds had the longest tail moments indicating a larger amount of enzyme restriction and less global DNA methylation than Lean birds. There was no effect of DTA on *Hpa*II-treated tail moments (Table 6.2, Model 6).

Table 6.2: Output from linear mixed effect models predicting HpaII-treated tail moments from experimental treatments and DTA in experiment 1. NB HpaII tail moment is log transformed to improve model fit. A * represents an interaction term.

Model Number	AICc	Dependent Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
4	11330.5	*Hpa*II Tail Moment	Amount*Effort *Msp*I Tail Moment	0.37 0.89	0.54 0.34	-0.15 (0.24) <0.01 (<0.01)	3220
5	11328.9	*Hpa*II Tail Moment	Amount Effort *Msp*I Tail Moment	4.82 2.60 0.91	0.03* 0.11 0.34	0.28 (0.12) 0.20 (0.12) < 0.01 (<0.01)	3220
6	9868.6	*Hpa*II Tail Moment	DTA *Msp*I Tail Moment	0.33 0.86	0.57 0.35	-0.12 (0.37) < 0.01 (<0.01)	2735

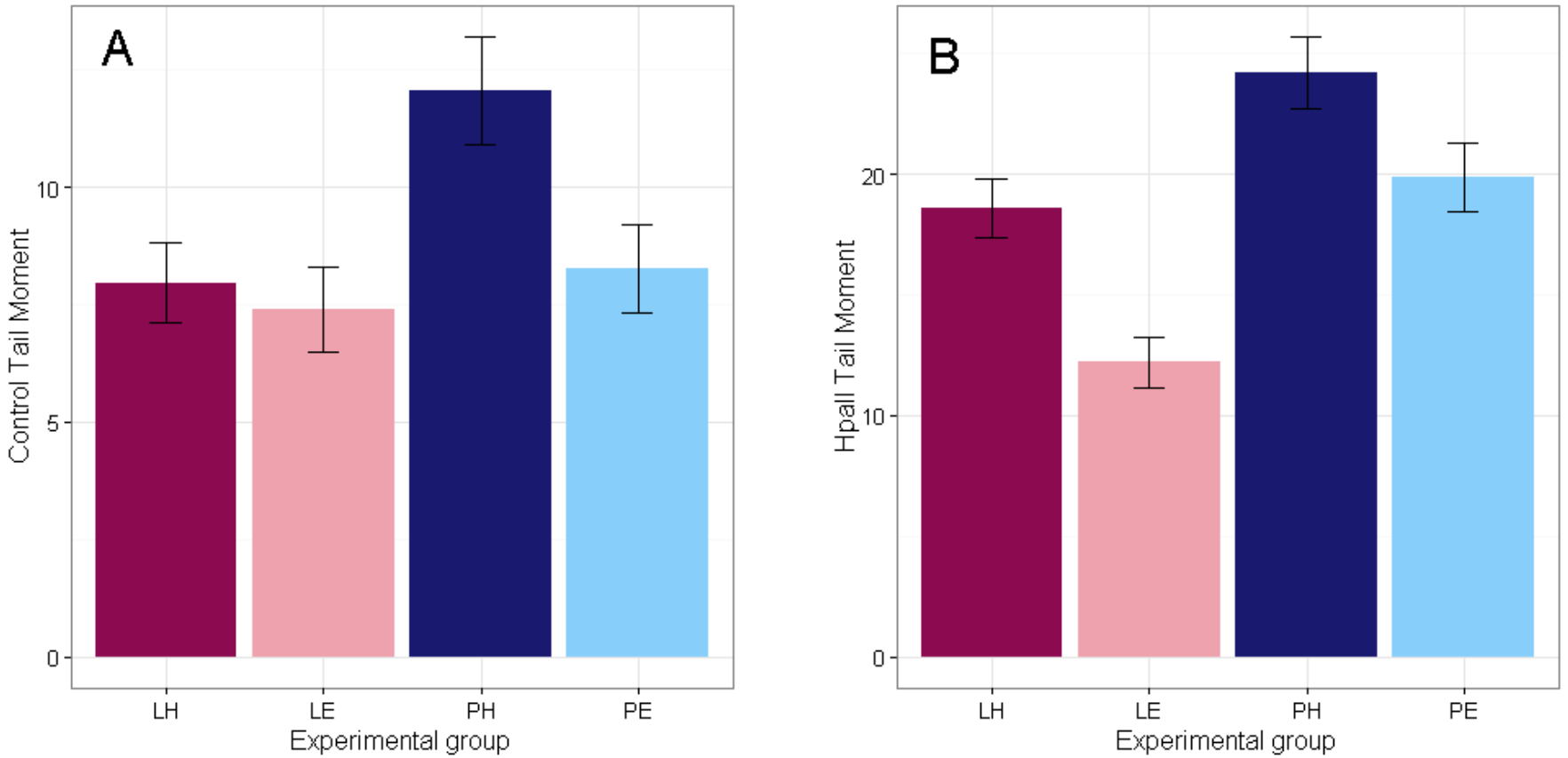

Figure 6.2: A) Average tail moments for control samples in experiment 1. B) Average tail moments for HpaII-treated samples in experiment 1. Data come from 32 starlings in 4 different developmental treatment groups (LH = Lean-Hard, LE = Lean-Easy, PH = Plenty-Hard, PE = Plenty-Easy). Tail moment was defined as the product of the tail length and tail intensity. Bars represent the mean tail moment, with error bars ± 1 SE of the mean of the scored comets. As a reminder in panel A), longer tail moments are indicative of more fragmented or damaged DNA. As a reminder in panel B), longer tail moments are indicative of more fragmented DNA due to a higher level of enzyme restriction. Therefore larger bars represent smaller quantities of DNA methylation.

6.3.2 Experiment 2

Experiment 2 refers to the comet assay conducted on blood samples taken from birds when they were approximately 18 months of age.

6.3.2a Control Tail Moments

Developmental treatments and DTA were used in mixed effects models for control tail moment data in experiment 2. There was a significant effect of Amount on control tail moments in samples taken from birds at 18 months of age (Table 6.3, Model 8; Figure 6.3a). Here, Plenty birds had significantly shorter tail moments than Lean birds in the control samples, indicative of less DNA damage. There was no effect of DTA on control tail moments in experiment 2 (Table 6.3, Model 9).

Table 6.3: Output from linear mixed effect models predicting control tail moments from experimental treatments and DTA in experiment 2. NB Control tail moment is log transformed to improve model fit. A * represents an interaction term.

Model Number	AICc	Dependent Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
7	5661.5	Control Tail Moment	Amount*Effort	< 0.01	0.92	-0.01 (0.13)	3026
8	5650.5	Control Tail Moment	Amount Effort	4.31 0.37	0.04* 0.54	-0.15 (0.07) -0.04 (0.07)	3026
9	5051.6	Control Tail Moment	DTA	< 0.01	0.97	< 0.01 (0.19)	2623

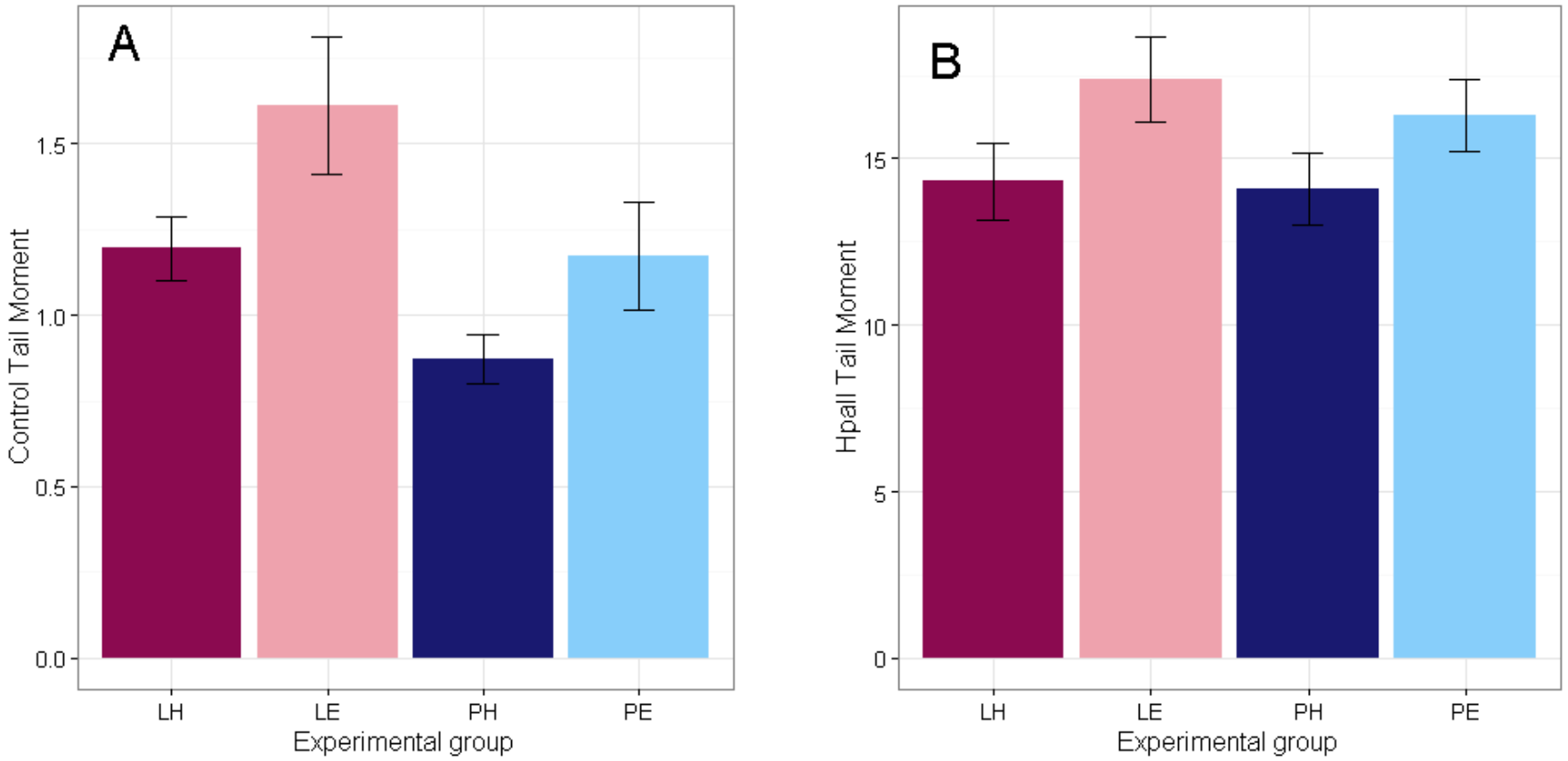

Figure 6.3: A) Average tail moments for control samples in experiment 2. B) Average tail moments for HpaII-treated samples in experiment 2. Data come from 30 starlings in 4 different developmental treatment groups (LH = Lean-Hard, LE = Lean-Easy, PH = Plenty-Hard, PE = Plenty-Easy). Tail moment was defined as the product of the tail length and tail intensity. Bars represent the mean tail moment, with error bars ± 1 SE of the mean of the scored comets. As a reminder in panel A), longer tail moments are indicative of more fragmented or damaged DNA. As a reminder in panel B), longer tail moments are indicative of more fragmented DNA due to a higher level of enzyme restriction. Therefore larger bars represent smaller quantities of DNA methylation.

6.3.2b *Hpa*II Tail Moments

There were no effects of Amount, Effort nor DTA on *Hpa*II-treated tail moments in experiment 2 (Table 6.4; Figure 6.3b).

Table 6.4: Output from linear mixed effect models predicting HpaII-treated tail moments from experimental treatments and DTA in experiment 2. NB HpaII tail moment is log transformed to improve model fit. A * represents an interaction term.

Model Number	AICc	Dependent Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
10	10259.3	*Hpa*II Tail Moment	Amount*Effort	2.01	0.16	-0.04 (0.21)	3013
			*Msp*I Tail Moment	0.03	0.85	< 0.01 (<0.01)	
11	10257.3	*Hpa*II Tail Moment	Amount	0.10	0.75	0.03 (0.10)	3013
			Effort	1.51	0.22	-0.13 (0.10)	
			*Msp*I Tail Moment	1.98	0.16	< 0.01 (<0.01)	
12	8931.9	*Hpa*II Tail Moment	DTA	0.24	0.62	-0.13 (0.25)	2611
			*Msp*I Tail Moment	1.47	0.23	< 0.01 (<0.01)	

6.3.3 Repeatability and Effects of Time

Blood samples collected from 6 randomly selected birds for experiment 1 were retested using the comet assay at the time point of experiment 2 to test the repeatability of data generated by the assay (discrepancies could be either due to experimental inaccuracies, or by the scoring technique used by the experimenter). Two-way ANOVAs with bird and time point (1 or 2) as factors were used to assess differences between the means of control tail moments, *Hpa*II-treated moments and *Msp*I-treated moments from the two comet assays. We found no significant effect of time point for any of the dependent variables, indicating that the data generated from the comet assay is repeatable (Table 6.5). Individual repeats were significantly correlated for the control and *Hpa*II treatments (control: r > 0.99, *P* < **0.01***; *Hpa*II: r = 0.98, *P* < **0.01***), but not for *Msp*I treatments (*Msp*I: r < 0.01, *P* = 0.99).

Table 6.5: Output from two-way ANOVAs predicting the effect of individual bird and time point on tail moments from control, HpaII-treated and MspI-treated samples used in the comet assay. A * represents an interaction term.

Dependent Variable	Fixed Predictors	F Value	*P*
Control Tail Moment	Bird	32.73	< 0.01*
	Time Point	1.03	0.31
	Bird*Time Point	0.24	0.95
***Hpa*II Tail Moment**	Bird	38.31	< 0.01*
	Time Point	0.11	0.74
	Bird*Time Point	0.34	0.89
***Msp*I Tail Moment**	Bird	1.03	0.40
	Time Point	1.70	0.19
	Bird*Time Point	0.99	0.42

Use of the same individuals in the running of experiment 1 and experiment 2 allowed us to detect how global CpG DNA methylation profiles can change over time. Table 6.6 shows that experiment (1 or 2) had a significant effect on all comet assay variables, with data differing between the two comet assays. The average tail moments for control, *Hpa*II-treated and *Msp*I-treated samples in experiment 1 were 1.2, 18.6, and 43.0 respectively. In experiment 2, the average tail moments were 5.2, 15.5 and 105.9.

Table 6.6: Output from linear mixed effect models predicting tail moments from experiment (1 or 2). Tail moment is log transformed to improve model fit.

Model Number	AICc	Dependent Variable	Fixed Predictors	LRT	*P*	B (± SE)	N
13	17303.8	Control Tail Moment	Experiment	365.94	< 0.01*	-0.48 (0.02)	6252
14	22001.4	*Hpa*II Tail Moment	Experiment	6.58	0.01*	0.09 (0.04)	6233
15	20745.7	*Msp*I Tail Moment	Experiment	2397.9	< 0.01*	1.78 (0.03)	6216

6.3.4 Experiment 3

Experiment 3 refers to the ELISA conducted on blood samples taken at 4 months of age. The mean level of global DNA methylation (5-mC) was 0.74% ± 0.37 (mean ±SD). There were no effects of developmental treatments (Figure 6.4) nor DTA on level of methylation as calculated by the ELISA kit (Table 6.7). As reported earlier, replication between duplicates was poor, with a CV of 110.91%.

Table 6.7: Output from linear mixed effect models predicting global DNA methylation as calculated in experiment 3. A * represents an interaction.

Model Number	AICc	Dependent Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
16	39.8	ELISA Methylation	Amount*Effort	0.19	0.66	-0.11 (0.27)	32
17	36.9	ELISA Methylation	Amount Effort	0.03 1.39	0.87 0.24	0.02 (0.13) -0.15 (0.13)	32
18	30.1	ELISA Methylation	DTA	0.01	0.91	0.03 (0.30)	32

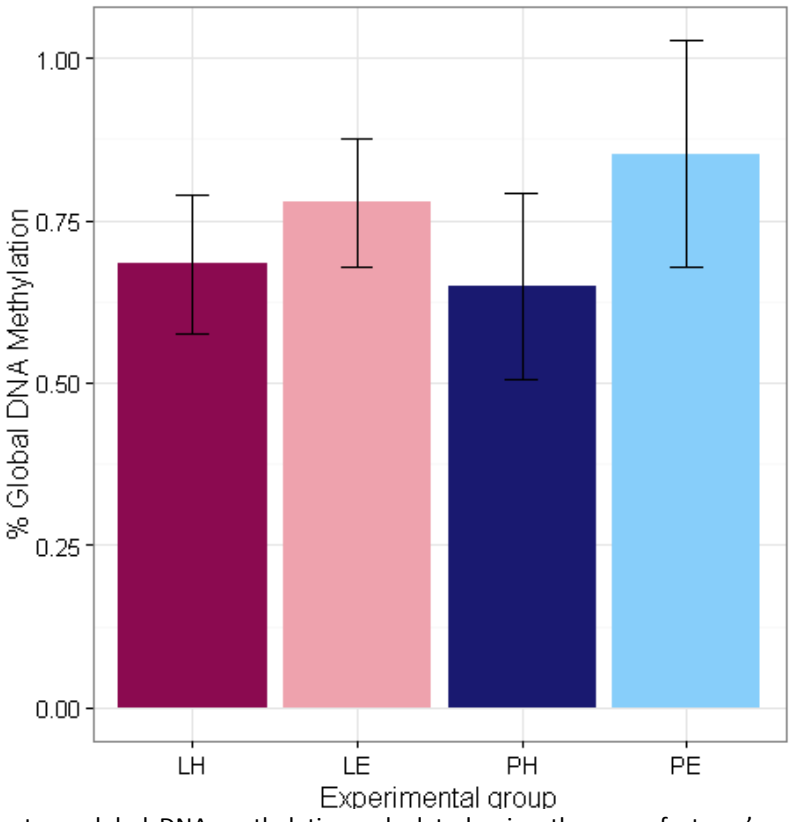

Figure 6.4: Percentage global DNA methylation calculated using the manufacturer's guide of the ELISA kit in experiment 3 for 32 starlings raised in 4 different treatment groups (LE = Lean-Easy, LH = Lean-Hard, PE = Plenty-Easy and PH = Plenty-Hard). Bars represent the mean percentage global DNA methylation, with error bars showing ± SE.

Neither *Hpa*II-treated tail moments nor *Msp*I-treated tail moments predicted methylation data as calculated by the ELISA kit (Table 6.8).

Table 6.8: Output from linear mixed effect models predicting global DNA methylation as calculated in experiment 3 from the mean of HpaII tail moments and the mean of MspI tail moments as calculated in experiment 1.

Model Number	AICc	Dependent Variable	Fixed Predictors	LRT	*P*	B (± SE)	n
19	37.8	ELISA Methylation	*Hpa*II Tail Moment	0.49	0.49	< 0.01 (<0.01)	32
			*Msp*I Tail Moment	< 0.01	0.99	< 0.01 (<0.01)	

6.4 Discussion

6.4.1 Summary of Results

The aim of this study was to both validate the use of the comet assay in measuring global DNA methylation and to determine whether differential experience of two different forms of early-life adversity could permanently affect DNA methylation profiles in the European starling. We measured two different aspects of methylation patterns. First, using blood samples taken from birds at 4 (experiment 1) and 18 months of age (experiment 2), we used a modification of the comet assay (Wentzel et al. 2010) to assess if exposure to methylation-sensitive restriction enzymes would show different digestion patterns, indicative of different levels of methylation at CpG sites across the genome. Second, using extracted genomic DNA from blood samples taken at 4 months of age, we used an ELISA kit to assess percentage levels of global DNA methylation expressed as the amount of 5-mC in comparison to other bases across the genome (experiment 3).

We found the comet assay to be repeatable over time, with repeated samples producing similar methylation data when measured a year later. In experiment 1, we showed that there was no difference in basal DNA damage due to developmental treatment or DTA, however there was a significant effect of Amount on *Hpa*II-treated tail moments. At 4 months of age, Lean birds had shorter tail moments than Plenty birds, indicative of less enzyme restriction and more global DNA methylation. We found that data between experiment 1 and experiment 2 were not comparable and that methylation profiles had changed over time. By experiment 2, the differences in global DNA methylation due to Amount had dissipated, however Amount now had an effect on levels of DNA damage. Here, Lean birds had greater comet tail moments than Plenty birds with respect to control

samples, indicating that Lean birds had more DNA damage than Plenty birds at 18 months of age. We found that neither of our treatment groups, nor DTA, significantly affected overall 5-mC methylation as predicted by the ELISA kit. Finally, there was no association between the data collected from the comet assay (experiment 1) and the ELISA kit (experiment 3).

6.4.2 Experimental Considerations

We validated the use of the comet assay in several ways. First, we found that in both experiment 1 and experiment 2, a significant increase in tail moments was detected after digestion with *Hpa*II and a further increase after digestion with *Msp*I when compared to the control samples (as seen in Wentzel et al. 2010; Wasson et al. 2006; Lewies et al. 2014). We also showed that the comet assay was repeatable over time, by showing similar methylation profiles for 6 birds analysed a year apart. Therefore, we believe that we have validated the use of the comet assay as a measure of global DNA methylation in European starling RBCs and the results discussed below are reliable. We showed that only very small quantities of blood are required to generate useable data. Blood samples are easy to collect and to store, and can be taken at multiple time points from one individual. It is worth mentioning that a significant increase in tail moments of *Msp*I-treated samples was seen between experiment 1 and experiment 2. We also found no correlation between individual *Msp*I-treated samples that were technically replicated (although there was no significant difference between the means of these replicates). This could suggest that there may have been a problem with the *Msp*I reagent in experiment 2, which could be related to long-term storage of the enzyme. However, these differences were not great enough to detect differences between the technical replicates.

When analysing *Hpa*II- and *Msp*I-treated tail moments, Wentzel et al. (2010) recommends the calculation of percentage global DNA methylation at CpG sites using a ratio of tail intensity in *Hpa*II- and *Msp*I-treated samples using the formula (100 - (*Hpa*II/*Msp*I)*100%). We believe that our approach using raw tail moment data in linear mixed effect models is a more sensible method of analysis, as opposed to generating a percentage methylation figure. By using the mean of the *Msp*I-treated tail moments as a covariate in our analysis of *Hpa*II-treated tail moments, this allows us to incorporate all of the data available to us, whilst avoiding any assumptions about how to construct summarising ratios. Our approach is more sensitive to subtle changes in levels of global CpG methylation and, whilst not giving absolute quantities of methylation, allows us to detect slight but significant changes in methylation with respect to early-life treatment.

6.4.3 Global DNA Methylation Profiles and Effects over Time

In experiment 1, we found that Amount (the absolute quantity of food received as a chick) significantly affected tail moments of *Hpa*II-treated samples. This indicates that this treatment had a significant effect on the activity of the methylation-sensitive enzyme *Hpa*II and ultimately on levels of CpG methylation profiles in the birds at four months of age. Tail moment data showed that Lean birds (those who received 73% *ad lib*) had significantly higher levels of methylated DNA than Plenty birds (those who received *ad lib*). We predicted that birds from adverse early-life conditions would have different levels of global DNA methylation. In support of this prediction, our findings indicate that various levels of early-life adversity imposed in our developmental manipulation have moderated global CpG methylation profiles, as evidenced from significant differences in *Hpa*II-treated tail moments between experimental treatment groups. It is difficult to say with our findings if we have found Amount to cause significant global hypo- or hyper-methylation relative to wild birds, as we do not have a control group as a reference point. However, the differences in methylation profile could leave birds from certain treatment groups more susceptible to disease in the future.

Our results are consistent with previous mammalian studies linking DNA methylation profiles to stress exposure early in life (Weaver et al. 2004; Tobi et al. 2014; Borghol et al. 2012). In our birds, food restriction increased CpG methylation later in life (also seen in Essex et al. (2013) - a human study of early-life adversity). However, a number of studies conversely report decreased global methylation following stress exposure as juveniles (Anier et al. 2014) or in adulthood (Rodrigues et al. 2015). We did not find any effect of Effort on DNA methylation profiles. It is likely that different types of early-life adversity can lead to hyper-methylation or hypo-methylation, however the exact mechanisms responsible for these changes are still unknown.

The absolute quantity of food received over the nestling period (Amount) appears to have had a greater effect than Effort in altering global DNA methylation profiles. Many examples show that nutrition can have drastic effects on epigenetics and overall behaviour and physiology (Vucetic et al. 2010; Altmann et al. 2012). Jousse et al. (2011) showed that having a restricted diet *in utero* in mice resulted in permanent hypo-methylation in the leptin gene promoter, alongside reduced levels of body fat and a higher overall food intake. The mechanisms by which nutrition itself could influence methylation patterns are well reviewed in Zhang (2015). Briefly, the methyl-donor required for DNA methylation is S-Adenosylmethionine (SAM) (Loenen et al. 2006). SAM is synthesised from precursors gained through diet including folate, betaine and vitamins B_2, B_6 and B_{12} (Mckay & Mathers 2011). Without adequate levels of SAM, the efficiency of methyl-donor enzymes is reduced and could lead to lower levels of global methylation. In our birds however, overall levels of methylation were lower

for birds who had received the largest quantities of food (Plenty birds). Our results suggest it possible that lower levels of food could instead hinder demethylation processes, leading to higher levels of DNA methylation genome wide.

In humans, restricted nutrition *in utero* has been linked with both hyper- and hypo-methylation of various loci in later life (Heijmans et al. 2008; Waterland et al. 2010). It is therefore not surprising that Plenty and Lean birds could show long-lasting methylation discordance due to differences in quantity of food received as a chick. In a comprehensive analysis, van Straten et al. (2010) found 137 areas of the mouse genome to be hyper-methylated following protein restricted diets, and 145 areas hypo-methylated, when compared to control diets, suggesting that areas of the epigenome differ in their sensitivity to food availability. By examining DNA methylation in these birds, we have the benefit of being able to study how these levels may change in the future, how specific regions of the methylated genome are affected by our treatments and any long-term physiological or behavioural effects.

We found that *Hpa*II-treated tail moments significantly decreased between experiment 1 and experiment 2 (representative of increased methylation over time) and that by experiment 2 (18 months of age), differences due to Amount in global DNA methylation profiles had disappeared. In humans, epigenetic marks of early-life adversity have been shown to persist into adulthood, with some studies finding methylation differences in individuals as old as 60 (Heijmans et al. 2008). In rats, exposure to maltreatment led to lifelong changes in methylation of the *BDNF* gene that were also passed down to offspring (Roth et al. 2009). In our cohort, it is possible that permanent changes to gene-specific methylation are masked by areas of the epigenome unaffected by nutritional restriction. As an individual ages, it is thought that the overall level of global DNA methylation decreases (Wilson & Jones 1983), however various regions have been shown to be hyper-methylated with age (Tra et al. 2002). Talens et al. (2010) showed that in 34 individuals, five of eight genetic loci investigated were stable over time with respect to methylation pattern. Therefore global DNA methylation changes over time are difficult to interpret without additional gene-specific studies to accompany them.

6.4.4 DNA Damage

We were able to detect DNA damage due to treatment differences by the time the birds were 18 months old (experiment 2). Here, birds who had restricted diets as chicks (Lean birds), had more DNA damage when compared to Plenty birds. Interestingly, these differences were not detectable at 4 months of age. It has been shown that exposure to stress can lead to increased DNA damage (Fischman et al. 1996; Flint et al. 2007). Tarry-Adkins et al. (2008) conducted a study assessing the effect of maternal protein restriction on DNA damage in rat pups. Using groups of restricted (offspring

of control-fed dams, nursed by restricted-protein dams), recuperated (offspring of restricted-protein dams, nursed by control-fed dams) and control rat pups (offspring of and nursed by control-fed dams), the authors demonstrated there was no DNA damage that could be attributed to treatment differences when measured at 3 months. However, at 12 months, increased levels of DNA damage were seen in the recuperated group. Similar to our experiment, individuals experiencing a restricted juvenile diet followed by a fast return to normal levels showed greater levels of DNA damage later in life. The authors speculate that rapid catch-up growth experienced by the recuperated offspring may hinder antioxidant defence capacity. Previously in this cohort of birds, Nettle et al. (2017) showed that oxidative damage was greater in Lean-Hard birds (those who had experienced the least amount of food for the most begging effort) at 56 days of age. We propose that this detected damage was directly due to the physical restrictive effects of the adverse manipulation, with our results from the comet assay capturing long-term changes in DNA damage due to enduring modifications to DNA repair systems.

6.4.5 ELISA

Using the ELISA kit, we found no effect of treatments or DTA on percentage global DNA methylation overall, however the assay had very poor replication between duplicates. Therefore the data presented here must be interpreted with caution. The results from the ELISA kit did not correlate with our comet analysis. There are several reasons why the two assays may not correlate. First, the ELISA kit measures the absolute quantity of 5-mC in the genome relative to other bases. We found an average 5-mC% of 0.74% in our starling RBCs. In humans, 5-mC levels are thought to be approximately 1% which corresponds to the results we detected. The majority of methylation in vertebrate somatic tissue occurs at CpG sites, with methylation at other bases thought to be rare, but still present (Schultz et al. 2015). The function of methylation at CpH (non-CpG) sites is still fairly unclear, however it may be important in demethylation processes (Fuso et al. 2010). The ELISA kit will detect 5-mC outside of CpG sites, however this will be missed in the comet assay. The use of the kit therefore gives a more comprehensive coverage of the genome, however the relevance of CpH methylation to gene expression is disputed and the benefit of the assay providing this additional information is not yet clear. Second, the restriction enzymes used in the comet assay have a tendency to digest sequences outside CpG islands, therefore full coverage of all CpG sites cannot be guaranteed (Wentzel et al. 2010). However, CpG islands in birds are usually found to be unmethylated (Li et al. 2011) and therefore our results gained from the comet assay are unlikely to be highly inaccurate.

It has been shown that DNA in hen erythrocytes has lower levels of methylation than other tissues such as liver, lung and white blood cells (Adams & So 1989). Adams and So found that, using

techniques that involved *Hpa*II enzyme restriction, approximately 3.28% of RBC and 3.95% of white blood cell DNA was methylated. Our levels of overall 5-mC are lower than those reported by Adams and So, however the 1989 experiment used the DNA of only one adult hen, as opposed to our 32 samples so it is hard to compare the accuracy of our results. Interestingly, an identical methylated DNA quantification kit showed global DNA methylation levels of ducklings to vary between 1.5-3% in peripheral blood, depending on pre-infection by the duck hepatitis virus (Xu et al. 2014). High levels of intra-assay variability in our ELISA study makes comparisons to other studies unwise. Instead, alternate methods of assessing global DNA methylation across all bases should be utilised.

6.4.6 Future Work

It is clear that genome-wide DNA methylation increases or decreases due to restricted diets are hard to interpret due to localised methylation changes in specific gene loci. Complementary studies looking at specific genes are also valuable and can offer further information into the biological processes specifically affected by DNA methylation changes (eg Weaver et al. (2004)). However, we argue that gene-specific analysis should be only considered with respect to global DNA methylation patterns. Guerrero-Preston et al. (2007) showed that changes in global DNA methylation levels may in fact precede changes in gene-specific methylation in the development of certain diseases such as liver cancer. Therefore a global DNA methylation measure for a species, as seen in this study, could provide information missed when conducting analysis into target genes. Gene-specific analysis itself is not without issue. For example, looking at target genes can be highly dependent on tissue-type specificity (Sant et al. 2012), which causes problems when collecting samples from living animals.

Interestingly, we found no effect of DTA on global DNA methylation profiles in any of our experiments. Several studies have linked DNA methyltransferases and telomere length in mammalian cells (Gonzalo et al. 2006), however we did not find any association between global methylation and DTA in this study. Wong et al. (2014) found a significant link between telomere length and global DNA methylation (measured in LINE-1 and Alu elements), therefore it is clear further investigation specifically designed to look at the relationship between telomere attrition and global DNA methylation is needed.

6.4.7 Conclusions

To our knowledge, this is the first study comparing the effects of two forms of adversity on methylation profiles in a long-lived bird. We have shown that different developmental treatments can lead to latent DNA damage and to differences in methylation at CpG sites that can be detected using the comet assay. However, this effect was not seen when looking at overall levels of 5-mC methylation.

It is likely that our manipulation specifically affected CpG site methylation, primarily found in promoter regions, and this knowledge was gained by the relatively novel use of measuring methylation with the comet assay. We have demonstrated that it is possible to tease apart effects of nutrition and more social forms of adversity and have shown that it is the former that has a more significant effect on global DNA methylation profiles, however these changes may not be as long-term and stable as once thought. Our results of overall 5-mC methylation gained by the ELISA kit are below what was expected when compared to the mammalian and avian literature, however the variation seen within this assay was exceptionally high, and therefore the results may not be accurate. Further studies on DNA methylation in birds using the comet assay would be useful for comparison. We have shown that the comet assay is a fast, cheap and sensitive way of measuring differences in DNA methylation in single cells, and that RBCs are an appropriate source of methylated DNA. However, RBCs have fast replacement rates and methylation changes in these cells may in fact be more representative of recent adverse experience. It is important to consider that with respect to the study of long-lasting effects of early development, more appropriate areas to target exist. For example, linking global DNA methylation in brain areas directly involved in adult physiology and behaviour may give more insight into the mechanisms by which developmental experience affects the phenotype.

The effect of early-life adversity on global DNA methylation is well-known across mammals, and now increasingly in avian species. Opening up new complimentary animal models whose *in ovo* development and ability to be hand-reared could facilitate distinctions between the effects of distinct pre- and post-natal adversity to be drawn. Long-lived species, such as the European starling allow for many behavioural and physiological measures to compliment DNA methylation data and can be studied long into the life of this animal.

6.5 References

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Chapter 7. Discussion and Conclusions

7.1 Thesis Summary

As described in the introduction to this work, there are many examples showing that phenotype can be modified by early-life environmental conditions (McGowan et al. 2009; Heim et al. 2008; Anda et al. 2006). Overall, we aimed to identify how different types of early-life adversity can affect adult behavioural and physiological phenotypes using the European starling as an avian model, and to consider the mechanisms that could shape this. To answer this, we used a unique developmental manipulation designed to dissociate the effects of overall quantity of food received (Amount – Plenty or Lean) from the begging investment required to obtain it (Effort – Easy or Hard) creating 4 experimental groups (Lean-Hard, Lean-Easy, Plenty-Hard, Plenty-Easy) (described in chapter 2). This manipulation allows us to tease apart effects of different types of early-life adversity, which has previously not been possible in other studies. The manipulation immediately affected many of the bird's characteristics, such as weight gain, skeletal size and developmental telomere attrition (DTA). In this thesis, we went on to test long-term effects of early life experience on the adult phenotype, with particular interest placed on traits involved in mood and emotional reactivity, such as Hypothalamic-Pituitary-Adrenal (HPA) profiles, anxiety-like and depressive-like behaviour. The creation of a cohort of animals that can be studied longitudinally, with repeated measures and with a detailed knowledge of past environmental experience, is critical to the field of early-life adversity, and as of yet, relatively unexplored in the literature. Finally, we explored DNA methylation as a potential mechanism that has been previously suggested to be involved in mediating the effect of the early-life environment on the adult phenotype. DNA methylation in birds is a novel and exciting topic, and we aimed to test if it was possible to measure global DNA methylation in birds using simple experimental assays, if profiles could indeed be shaped by early-life experience, and if DNA methylation could be considered a candidate for a possible mechanism by which adult phenotypes can be shaped.

7.2 CORT Profiles and the HPA Axis

In chapter 3, we measured Hypothalamic-Pituitary-Adrenal (HPA) profiles in our cohort of birds at two different time points (at 4 and 18 months of age). To do this, we took measurements of corticosterone concentrations (CORT) at baseline, and at 10 minutes and 15 minutes post-exposure to an acute

stressor. From this, we derived three variables; baseline CORT (circulating CORT within 3 minutes of the stressor), peak CORT (greatest concentration of CORT overall) and ΔCORT (a measure of negative feedback – the difference between CORT 30 minutes post-stressor and 15 minutes post-stressor). We found significant evidence to suggest that two aspects of the HPA axis (peak CORT and ΔCORT) can be shaped by early-life experience, however baseline CORT levels were unaffected. We predicted that early-life adversity would lead to a hyperactive stress response, however this was not what we initially observed.

At 4 months of age, we found that birds from Hard treatment groups (those who had experienced increased begging effort) had an attenuated stress response, with lower peak CORT and potentially a faster, more efficient negative feedback system (as indicated by a higher value of ΔCORT), seen particularly in the Plenty-Hard experimental groups. We speculated that this suppression of the HPA axis in developmentally stressed individuals may be adaptive and functions to protect from further HPA-activated damage (Kitaysky et al. 2003). We found evidence to suggest that effects of early-life adversity are not stable over time. By 18 months of age, peak CORT had decreased, consistent with studies that explain this by an increase in chronological age (Wilcoxen et al. 2011; Heidinger et al. 2006). Treatment effects were still detectable on ΔCORT, however with a few key differences. First, there was now an interaction of Effort by Amount (as opposed to a main effect of Effort at 4 months), showing that separate sources of adversity can affect the HPA axis using mechanisms that work over different timescales.

At 18 months, Plenty-Hard birds showed a prolonged stress response, and instead, Plenty-Easy birds had faster cessation of the HPA axis, as we had predicted. We speculated that this reversal could be evidence to show that early-life environmental adversity has detrimental effects on the HPA axis that are delayed until an animal is older. Indeed, Henry et al (1994) demonstrated that early restraint stress in rats was sufficient to prolong CORT secretion (equivalent to decreased ΔCORT in our experiment) post-exposure to novelty at 90 days, but these effects were not seen at 3 or 21 days of age. This is similar in humans, as the functioning of the HPA axis is not fixed at birth, and changes in cortisol reactivity occur throughout childhood (Gunnar & Donzella 2002). Our study shows that this may also be true in European starlings, with true effects of early-life adversity on the HPA axis not revealed until later in life. This is important for diagnosing alterations to the HPA axis in humans, as we have demonstrated that detrimental effects of early-life adversity may not manifest until the patient reaches adolescence or even adulthood.

An interesting result to come from our CORT profile experiments was that CORT measurements were not repeatable at different time points, having important implications for future research. This finding

highlights the fact that caution must be taken when correlating behavioural and physiological results taken from different time points, and that experimenters should always report this time difference. It is possible that HPA responses attenuate over time due to habituation to laboratory life, or to the capture procedure. Therefore, physiological data taken at one time point may not be an accurate representation of a stress response later in life. We would emphasise the need for further studies to take repeated CORT measures over the lifetime of a laboratory animal (a practice not often taken) to further investigate this. We found that repeatability of ΔCORT differed between treatment groups. Due to the design of our cohort (7-8 birds in each treatment group), we must be cautious in the interpretation of this result, as within-group analysis lacks statistical power. Therefore, repeatability of CORT measurements needs to be examined in a larger sample size of animals with a uniform developmental history to investigate this further.

There is a large body of literature showing that early-life adversity leads to long-term changes of the HPA axis. It is thought that disruption (particularly that which results in hyperactivity) to the HPA axis, can leave individuals susceptible to mood disorders (Arborelius et al. 1999). We have shown that early-life adversity can alter the HPA axis in European starlings, however it is difficult to conclude whether this leaves the system hyperactive, as further studies over the lifetime of this cohort are needed. It would be interesting to see if stable effects, particularly with respect to ΔCORT, emerge over the lifespan of the birds. Our research provides insight into the underlying HPA functioning of animals exposed to different sources of early-life adversity, and shows that the stress response could be a mechanism by which the early environment can leave an individual susceptible to stress-related disorders.

7.3 Anxiety-Like and Depression-Like Behaviour

In chapters 4 and 5, we detailed behavioural experiments that independently assessed anxiety-like and depressive-like behaviour in our starling cohort. In chapter 4, we exposed birds to a distress call (which we demonstrated was sufficient to generate an anxiety-like state in birds) and tested a suite of behaviours that represented different aspects of anxiety with respect to the independent introduction of two resources (a bowl of *ad lib* food or a water bath). As discussed in chapter 4, there were several important limitations to the interpretation of the results from this study. First, we found significant differences in exhibition of anxiety-like behaviour between the introduction of the bowl (experiment 1) and the water bath (experiment 2) that we could not rule out were due to experimental order effects. We also highlighted that lack of bathing may have made the birds more sensitive to cues of danger (as seen in Brilot & Bateson 2012), and therefore it was not possible to directly compare treatment differences between the two anxiety experiments. Finally, we were not able to create an

experiment in which we tested animals in a “stressed” and “non-stressed” condition due to limitations caused by the methods of introduction of the resource to the animal’s cage. All trials (with and without the distress call) included birds being exposed to sudden darkness and the experimenter putting their hands into cages. This introduces uncontrollable individual differences concerning how stressful each bird finds this process.

We found a large amount of evidence that early-life adversity can affect adult stress reactivity and increase anxiety-like behaviour. In terms of treatment differences, we showed that both Amount (the quantity of food received) and Effort (begging effort required to obtain this food) had significant independent and interactive effects on different measures of anxiety. Lean birds (those who had received approximately 73% *ad lib* diet) had a significantly longer freezing response post-exposure to a distress call, and Hard birds (those who had experienced increased begging effort) displayed more vigilant behaviour when feeding. Interestingly, Effort had a significant effect on anxiety-like behaviour regardless of exposure to the distress call, suggesting that increased begging effort had amplified underlying baseline anxiety, however a restriction in food significantly increased anxiety produced in response to an acute stressor. Fitting with this, early-life dietary restriction has been seen to increase anxiety-like behaviour in rodents (Jahng et al. 2007; Levay et al. 2008). It is often assumed that social measures of early-life adversity are the greatest risk factors for the development of anxiety. Here, we show that physical factors such as food shortages can also play a significant role in the etiology of psychological disorders, and should not be discounted when attempting to diagnose the causes of anxiety. Human studies investigating the correlation between anxiety disorders and early life often assess childhood adversity in terms of the social environment (such as abuse, aggression, traumatic experiences etc). Where possible, a record of physical factors such as childhood illness or periods of food unpredictability should be incorporated into human self-reported measures of adversity.

We found that both aspects of early-life adversity increased the expression of anxiety-like behaviour in our cohort, meeting our predictions. As discussed in chapter 4, this fits with findings from animal models of anxiety (Jahng et al. 2007; Levay et al. 2008; Andrews & Rosenblum 1994) and the human literature (Stein et al. 1996; Kuo et al. 2011; Portegijs et al. 1996). Our data show that the European starling should be considered a good candidate for an animal model of anxiety, and can be used to experimentally test causation of such diseases. We have shown that a fairly straightforward and short developmental manipulation has been sufficient to alter anxiety-like behaviour in the European starling, with both restricted food and increased begging effort having significant additive and interactive effects in increasing anxiety. It would be interesting to measure anxiety again in these birds as they age, as this would give an indication of the likely recovery time (if at all) from anxious

phenotypes mediated by early-life adversity, and could be applied to humans when treating this disease.

In chapter 5, we assessed expectation of reward and depression-like behaviour in adult birds. Here, we trained birds to associate a positive stimulus with a mealworm reward and a negative stimulus with nothing. We tested cognitive bias by presenting ambiguous stimuli intermediate to the two learned stimuli. As a reminder, birds that respond to the ambiguous stimuli in a manner similar to the positive stimulus are said to be more optimistic. Birds that respond in a manner similar to the negative stimulus are said to be more pessimistic. We found modest evidence to suggest that our developmental manipulation affected depression-like behaviour, with Hard birds (a subsection of the Effort treatment) showing more optimistic-like behaviour and an increased expectation of reward with respect to ambiguous stimuli, but only on the first presentation. This effect was not strong enough to last over 4 days of testing. In chapter 4, we argued that we believe the "first look" analysis gives a more insightful measure of cognitive bias, as it removes any effect of learning that the ambiguous trials do not offer a reward.

It is interesting to find that only Effort (and not Amount) had an effect on depressive-like behaviour, suggesting that social forms of stress may be more important in the etiology of this phenotype than nutritional shortages *per se*. This was not found to be the case with anxiety, where we found effects of both Amount and Effort on anxious phenotypes. We found that increased early-life adversity in the form of increased begging effort reduced levels of depression-like behaviour, which was unexpected. The literature generally agrees that an increase in adversity leaves an individual susceptible to the development of depression in humans (Heim et al. 2008; Sadowski et al. 1999; Parker et al. 1995; Kendler et al. 2002), however the animal literature provides mixed results (Bateson et al. 2015; Chaby et al. 2013; Brydges et al. 2012). We have shown that it is unwise to assume that early-life adversity will naturally lead to development of depression, and this should be taken into account when trying to identify the cause of depressive phenotypes in animals.

It is important to bear in mind the limitations of our interpretation into depression-like behaviour with respect to early-life adversity, as there were no statistically significant treatment differences detectable when looking at responses to ambiguous stimuli over the complete testing period. We went on to discuss that differences in learning capabilities (either due to genetic causes, or directly due to the developmental manipulation) may be responsible for masking intrinsic cognitive biases. We were able to show that there were differences in associative learning capabilities that could be attributed to the developmental manipulation, as Plenty-Easy birds were significantly faster to probe negative stimuli throughout the testing period. As the testing period involved the repeated

presentation of ambiguous stimuli, it is likely that different treatment groups learnt the unrewarded nature of these stimuli faster than others. Therefore, faster latencies to approach ambiguous stimuli could be interpreted as either an altered expectation of reward or a demonstration of faster learning capabilities. Previously, learning and memory have been shown to be impaired by early-life adversity (Kaplan et al. 2001; Nelson et al. 2007). Further cognitive studies into depression-like behaviour that do not require extensive training, learning or memory would remove this confusion.

We found striking differences between the effects of early-life adversity and the development of anxiety-like and depression-like behaviour in our birds. We showed that increased developmental stress can lead to more anxiety-like, but controversially, to less depression-like behaviour. This was unexpected, as the human literature often reports comorbidity of anxiety and depression (Moffitt et al. 2009) implying that a common mechanism is responsible. Liu et al. (2017) found that exposing rats to a single prolonged stress model (2 hours restraint, 20 minutes forced swim and 2-3 minutes of ether anaesthesia) during early-life led to anxiety-like behaviour at life stages equivalent to human adolescent and adulthood, however the development of depression-like behaviour was not identifiable until much later in life. We have shown that at approximately 6 months of age, depression-like behaviour is in fact decreased by early-life adversity, however by 22 months, increased anxiety-like behaviour could be identified and attributed to our developmental manipulation. A limitation of our interpretation lies in the fact that we did not conduct both analyses of anxiety-like and depression-like behaviour at each time point, and cannot confirm whether depression-like behaviour is replaced by anxiety, or if the two phenotypes develop independently of each other. It is possible that depression-like behaviour does not develop until later in life in birds, and therefore further behavioural studies in this cohort confirming this would be of interest. Our behavioural studies have important clinical relevance as they identify that there may be specific mechanisms responsible for the independent development of anxiety and depression, and it should not be assumed that the existence of one inevitably leads to the other.

7.4 Comparing Physiological and Behavioural Data

As well as exploring developmental treatment differences, we looked at the relationship between physiological CORT data and behavioural measures. As mentioned previously, a limitation of this is that we showed our CORT measurements to be unrepeatable across different time points. Whilst using CORT measures taken in as close a time as possible to the behavioural experiment (chapter 4 – CORT measured approximately 4 months previously, chapter 5 - CORT measured 1-3 months previously), we cannot be sure that HPA responsivity at the time of the behavioural studies is represented by the CORT data we collected. With hindsight, it would be more reliable to sample CORT concentrations at a closer

time point to the behavioural studies and use these data in our analyses. A further consideration that must be taken when looking at associations between our physiological and behavioural results is that data were not collected in response to the same acute stressors. For example, physiological CORT data was collected in response to a standardised capture-handle-restraint procedure, and anxiety-like behaviour post-exposure to a conspecific distress call. Pacak & Palkovits (2001) show that neuroendocrine responses to different stressors (immobilisation, cold, pain, hypoglycaemia and haemorrhage) can be heterogeneous. It is therefore important to consider that the HPA response measured when captured may not be fully representative of a response when exposed to a distress call. However, as both stressors are predator-specific, the disparity is not so great that the results are not comparable.

An increase in circulating baseline CORT has previously been associated with anxiety-like behaviour (Fan et al. 2014; Mitra & Sapolsky 2008), however studies suggesting CORT to have anxiolytic effects also exist (Albrecht et al. 2013; Heim & Nemeroff 2001). When comparing the CORT data collected in chapter 3 to anxiety-like behaviour, we showed several interesting results. We found that an increase in baseline CORT was associated with more anxiety-like behaviour, measured in terms of latency to move, vigilance and resource use, supporting the former examples. Surprisingly, peak CORT did not have a relationship with any measure of anxiety. However, it is important to state that the behavioural data was collected over 25 minutes post-stressor, and we have shown that CORT may not reach peak levels until 30 minutes. We also found an interesting result concerning ΔCORT and anxiety, with greater negative feedback and faster cessation of the stress response being associated with more anxiety-like behaviour. ΔCORT is a measurement gained between 15 minutes and 30 minutes post-stressor, so it is difficult to ascertain the biological relevance of the measurement of ΔCORT in this particular study. A behavioural study that looks at anxiety and vigilance post-30 minutes of a distress call would help to answer this. We found no relationship between CORT measured at 4 months and depression-like behaviour. In humans, decreased baseline CORT has been associated with increased optimism and a positive affective state (Lai et al. 2005; Endrighi et al. 2011), however we found no evidence to support this. We did, however, find CORT effects on learning behaviour, with greater ΔCORT being associated with faster and more reliable associative learning.

Generally speaking, the consensus in the literature is that a hyperactive HPA axis can lead to an increase in anxiety-like or depressive-like behaviour (Arborelius et al. 1999; Abelson et al. 2007). We found a small amount of evidence that agreed with this theory (increased baseline CORT correlating with more anxiety-like behaviour), however we have discussed several reasons within the relevant data chapters why the physiological results may not correlate with the majority of behavioural data in this case. As well as experimental issues, disparity between CORT data and behavioural data may be

due to the involvement of alternate mechanisms responsible for the production of anxious or depressive phenotypes. Many suggestions for a mechanism for the development of depression and anxiety have been put forward, such as a decreased GABAergic system, variation in serotonin transporters and hippocampal volume reduction (Möhler 2012; Holmes et al. 2003; Bremner et al. 2000). Further neurobiological studies are necessary to elucidate likely candidate systems for the development of mood disorders, and should not be restricted to tests of increased HPA activation. Finally, it is possible that other aspects of the HPA axis that were not measured in this thesis are better predictors of anxious and depressive phenotypes, such as GR and MR (mineralocorticoid receptor) expression or levels of adrenocorticotropin hormone (ACTH).

7.5 DNA Methylation as a Mechanism

In chapter 6 we explored a novel mechanism for how early-life adversity can mediate changes in the adult phenotype. We aimed to identify if early-life adversity has the capacity to alter global DNA methylation profiles in European starlings, and if these effects were persistent over time. To do this, we validated the use of an interesting modification of the comet assay. We found evidence to suggest that early-life adversity does indeed have the capacity to change global DNA methylation at 4 months of age, with Lean birds (those which experienced approximately 73% of *ad lib* food) showing less global DNA methylation than Plenty birds (those which experienced *ad lib* food). Interestingly, we found no effect of Effort on DNA methylation profiles, indicating that DNA methylation is altered by a nutritional and not a social measure of early-life adversity. Our results support other findings in the mammalian literature, linking a reduction in food to altered methylation later in life (Essex et al. 2013; Jousse et al. 2011; Heijmans et al. 2008; Waterland et al. 2010). The mechanisms responsible for this are as yet unknown, however we suggest that lower quantities of food could restrict demethylation processes and lead to higher levels of global DNA methylation. Further biochemical studies are needed to elucidate exactly how a reduction in food can lead to an increased amount of global DNA methylation. To investigate how DNA methylation can change over time, we repeat sampled 30 individuals at 18 months of age. At 18 months of age, treatment effects had dissipated and there was no significant difference in global DNA methylation between any of the experimental groups. Our results therefore indicate that epigenetic marks may not be stable throughout the life of an animal.

As mentioned in chapter 6, this study acts as an interesting investigative approach, and should be complimented with gene-specific methylation approaches, as early-life adversity has been previously shown to significantly alter methylation at specific loci (eg Sandovici et al. 2011). An obvious candidate system to explore relevant to the work presented here are genes and promoter regions involved in the HPA axis. In humans, McGowan et al. (2009) showed that the gene coding for the glucocorticoid

receptor (*NR3C1*) was under-expressed in suicide victims who had suffered child abuse when compared to controls with no such history. They also showed that these individuals had increased methylation at the *NR3C1* promoter, consistent with work on early-life stress and GR promoter methylation shown in animal models (Weaver et al. 2004). Therefore, an excellent complement to our work presented here would be the addition of methylation status on particular genes, beginning with information on the glucocorticoid and mineralocorticoid receptors. Another useful addition to the literature on avian DNA methylation would be to take blood samples from our cohort in response to different environmental conditions. For example, during different seasons or post-exposure to acute or chronic stressors during adulthood.

We have provided evidence that DNA methylation can be a mechanism by which the adult phenotype is shaped by the environment and is altered by the early-life environment. Animal studies show that early-life stressors (for example, maternal exposure to drugs, stress or toxins) can modify epigenetic marks at many regions across the genome (Jensen Peña & Champagne 2012; Monk et al. 2012; Kundakovic 2013). Our data add to the list of species that experimentally show that DNA methylation can be altered by stressors early in life. Complex illnesses such as cancer, diabetes and obesity have been identified as diseases in which susceptibility is increased when coupled with exposure to early-life stress, with the exact etiology yet to be uncovered. Therefore, we have shown that changes in DNA methylation could be responsible for the development of such disorders, and show a possible route for the development of treatments and cures. In chapter 6, we discussed that our cohort does not allow us to state categorically whether our developmental manipulation has increased or decreased global DNA methylation with respect to wild animals. Instead, a fairly simple but comprehensive addition to this study would be to collect data from a large sample of wild European starlings to gain a baseline level of global DNA methylation to compare to our data.

7.6 Telomere Attrition

Throughout this study, we found consistent differences in the effects of developmental treatment and telomere attrition (also seen in Bateson et al. 2015). We found no effects of DTA throughout this thesis, with the exception of chapter 4 and anxiety-like behaviour. Here, we found that birds with greater DTA showed more anxiety-like behaviour upon exposure to the stress call when they had been deprived of bathing for 24 hours. The human literature also shows correlations between shorter telomeres and anxiety disorders (Simon et al. 2006; Okereke et al. 2012; Needham et al. 2015), however it is still unknown why this may occur. Studies such as ours add to the literature that discusses the role of accelerated ageing and the development of mood disorders such as anxiety. However, we found no effect of DTA on CORT profiles, depression-like behaviour or global DNA methylation.

We previously showed that DTA was accelerated in birds that had experienced the lowest amount of food and the highest begging effort (Nettle et al. 2017; Chapter 2), with previous studies suggesting that perhaps DTA is a greater predictor of early-life stress than developmental treatment (eg Bateson et al. 2014). However, these studies refer to alternate developmental manipulations, unable to tease apart the effects of restricted nutrition and begging effort. It is worth noting that these manipulations sometimes found opposite effects of DTA and experimental treatment, as seen in this body of work (Bateson et al. 2015). As we consistently found stronger effects of developmental treatment than telomere attrition throughout this thesis, it is possible that we have created a developmental manipulation that is a stronger predictor of early-life adversity than DTA.

7.7 Adaptive Responses to Early Environmental Signals

Throughout the thesis, the focus of the work has been to relate the findings to conditions present in the study of human psychology and the development of disease. However, the topic of early-life adversity is relevant to many fields, including adaptation and plasticity. The results presented here can therefore be discussed in multiple contexts. It can be advantageous, in fitness terms, for individuals to show particular traits given their developmental histories. This can be mediated by developmental plasticity (the capacity of the same genotype to produce different phenotypic outcomes depending upon environmental cues during development) (Stearns 1989).

In chapter 3, we showed that increased early-life adversity could affect adult CORT profiles long after the developmental manipulation had ended. Our results show that the HPA response in starlings is indeed plastic, both in response to different developmental environments, and also over time as an individual ages. We discussed in this chapter that it is possible that our early-life developmental manipulation provides environmental cues that lead to the development of different HPA responses.

In chapter 4, we found that increased early-life adversity could lead to a more anxious adult phenotype, potentially leaving an individual more susceptible to developing a pathological anxiety disorder. However, the development of such a phenotype could also be thought of as being adaptive. Anxiety-like phenotypes include behaviour such as increased vigilance and attentional bias to danger, and physiological changes to increase 'fight or flight' responses. This can be highly adaptive in an environment that consists of frequent threatening situations. Our early-life treatments could provide an individual with information about the adult environment, and could influence the development of a more anxious phenotype if this would increase the likelihood of survival. However, it is yet to be determined if information about developmental food supply can predict the most adaptive phenotype in the adult environment in terms of anxiety and vigilance.

Alternatively, it is possible that the developmental manipulations are providing cues about adult food supply. The behaviours seen in chapter 4 that we have interpreted as a more anxious phenotype (increased scanning of the environment when eating and increased latency to begin moving following the introduction of a food resource) could instead be considered as a difference in food motivation mediated by the experimental treatments. Here, it is possible to interpret increased anxiety-like behaviour (caused by increased early-life adversity) as reduced food motivation or hunger. Again, this may be adaptive. Both the Lean and the Hard treatments provide information that the nutritional environment is poor (either through there being only small quantities of food, or by being unpredictable in its delivery). It would make adaptive sense for birds from these treatment groups to expect potential food rewards to be of less value than Easy or Plenty groups. Therefore, these birds may be less motivated to approach and use food resources when presented after a potential threat. However, this experiment did not show any significant differences in how birds used the food resource directly, and so could not establish definitively if there were differences in adult food motivation in this cohort. We have also previously shown that increased early-life adversity, contrary to the results found in chapter 4, lead to birds that had naturally hungrier phenotypes and were in fact more food motivated (Bloxham et al. 2014).

In chapter 5, we showed that Hard groups were, contrary to our predictions, in a more positive affective state. We concluded that these individuals were less likely to develop a depression-like disorder. Again, this could be argued as being an adaptive response elicited by cues in the early environment, as exposure to early-life adversity could function to protect the adult from developing a depressive phenotype. However, this is not what is generally seen in the human literature (Parker et al. 1995; Sadowski et al. 1999; Kendler et al. 2002; Heim et al. 2008). Similar to chapter 4, the results presented in this experiment could be explained in contexts such as risk, impulsivity and food motivation. Hard birds, having had experience of unrewarded nest visits, may have been developmentally programmed to combat this by intrinsically increasing their adult expectation of reward. This would be adaptive in an environment that had unpredictable feeding opportunities (similar to the Hard treatment groups), as birds would be more likely to investigate potential food sources and ultimately increase their chances of finding food. Easy birds, having learnt that food is always available or in sufficient quantities, do not require this adaptation and are less likely to interpret unknown ambiguous (and potentially dangerous) stimuli as food sources.

The study of early-life adversity and phenotype adaptation is complex, and easily interpreted from both sides. On the one hand, it is possible to argue that an adverse developmental environment predicts the conditions to be faced in the adult environment, and this allows an animal to ‘match’ the most adaptive phenotype. This is the best fit for the data presented in chapter 4. Birds exposed to

adverse environments showed more anxious-like phenotypes in adulthood, potentially in an attempt to match their behaviour to an unpredictable adult environment. Here, more anxiety-like behaviour is more adaptive. The other hypothesis, more appropriate for the data seen in chapter 5, states that a poor early environment allows the development of a phenotype that is more robust to an adult world in general (this may match the developmental environment or it may not). Birds exposed to more early-life adversity could be naturally protected against developing a depressive phenotype in adulthood. Both theories are adaptive, however differ in the interpretation of whether early life predicts the adult environment. Though this thesis has been primarily focused on human health and disease, it does raise interesting questions about adaptation and the role that early-life adversity can play.

7.8 Conclusions

This thesis set out to investigate if early-life adversity could affect HPA, depression-like and anxiety-like phenotypes in adult animals. We found evidence to suggest that all of these characteristics could be significantly changed by early environmental conditions, however the nature of these modifications were not necessarily in the direction predicted. We have shown that a poor developmental environment does not always lead to a disadvantaged animal, particularly in adolescence, in terms of HPA profiles and depression-like behaviour. However, in adulthood, early-life adversity was significantly associated with increased anxiety-like behaviour and a prolonged stress response. We showed that both nutritional restriction and begging effort can have significant independent effects on different components of HPA profiles, anxious and depressive phenotypes. This suggests that different sources of adversity could target specific mechanisms. We aimed to investigate if changes to the HPA axis were associated with the expression of depression-like and anxiety-like behaviour, but only found limited evidence to support this hypothesis. Finally, we investigated the role of early-life adversity in the shaping of DNA methylation profiles. We found significant effects of one of our developmental treatments on global DNA methylation, and concluded that methylation was indeed a mechanism through which the early environment could affect the phenotype. However, as these changes resolved with age, it is still unknown whether DNA methylation changes during development can lead to lifelong changes to the adult phenotype. Particular importance was placed upon the quantity of food received as a nestling, with a restricted diet leading to higher levels of DNA methylation overall.

There are several key implications of the work presented here, in terms of the developmental manipulation, experimental methodology and to the study of early-life adversity. In terms of the manipulation, we have shown many benefits to using the cohort of animals described here.

Throughout this work, we have demonstrated the importance of creating adversity paradigms that can dissociate effects of physical and social stressors. Throughout the work presented here, we have consistently shown independent effects of both Amount and Effort, as well as interactions between the two treatments. Studies that cannot tease apart these different aspects of early-life adversity are missing a large amount of data relating to the different pathways and mechanisms that can be targeted by types of adversity. It is crucial to use such developmental manipulations to create cohorts of animals that can be studied over time, in multiple behavioural, cognitive and physiological experiments. The benefit of having individuals in which there is a complete and well-controlled life history which can be studied as the animal's age is crucial when relating work to the development of disease in a long-lived species such as humans. Through this cohort, it has been possible to study many different effects of early-life adversity, how they change with age, and how these measures correlate with one another. This has allowed us to create a much more comprehensive and insightful study concerning the effects of early-life adversity on the adult phenotype.

7.9 References

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