January 2013

Physiological and Behavioral Mechanisms of Range Expansion in the House Sparrow (Passer domesticus)

Andrea Lyn Liebl

University of South Florida, aliebl@mail.usf.edu

Follow this and additional works at: http://scholarcommons.usf.edu/etd

Part of the Ecology and Evolutionary Biology Commons

Scholar Commons Citation
Physiological and Behavioral Mechanisms of Range Expansion in the House Sparrow

(*Passer domesticus*)

by

Andrea Lyn Liebl

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Integrative Biology
College of Arts and Sciences
University of South Florida

Major Professor: Lynn B. Martin, Ph.D.
Jason R. Rohr, Ph.D.
Christina L. Richards, Ph.D.
Toru Shimizu, Ph.D.

Date of Approval:
June 12, 2013

Keywords: Invasive species, Glucocorticoids, Exploration, Memory

Copyright © 2013, Andrea Lyn Liebl
DEDICATION

I am indebted to many people for their help in this culmination of this Ph.D. First, I want to thank my family, particularly my mom who instilled a passion for science into me by taking me to “the beach” every year and answering my incessant questions about wildlife, the ocean, and anything I saw. I am also grateful of my whole family for always encouraging me to pursue my dreams, no matter how difficult- without their support this would not have been possible.

At USF, I would like to thank Marty for bringing me into the lab and helping develop my scientific abilities, both technically and intellectually, as well as funding a great deal of my research and encouraging me to persevere. My committee members, Jason Rohr, Christina Richards, and Toru Shimizu, have been a great source of support, helping with statistical analyses, acting as a sounding board, and casting a critical eye over my research. My lab mates (Amber Brace, Martyna Boruta, Courtney Coon, Holly Kilvitis, Josh Kuhlman, Cris Ledon-Rettig, and Brittany Sears) have offered me great support, technically, intellectually, and emotionally, and I am grateful for that. I am also indebted to the countless other friends at USF and elsewhere for their support through the years including (but not limited to): Chris Anderson, Kate Golden, Jess Hok, Jen Wimmer, Jen Hoskins, Joz Mills, Kristin Svorinic, the Rohr lab, and Nate and Lindsay Goddard. Additionally, many undergraduate students have provided considerable help throughout my time at USF, including time in the lab, time in the field, and discussion; I
am grateful for each of their contributions and hope they also gained something from their experiences with me. Specifically, I would like to thank Jen Alam, Zahra Alipour, Melinda Fang, Ashley Garringer, Chloe Jofeson, Laura Kidd, Brittany Leigh, Steffanie Munguia, My Nguyen, Jaymin Patel, Christina Ruiz Lorenzo, Evelyn Schmidt, Roanak Shah, Alexandra Sierra, Nhan Tu, Alex Urban, and Desirae Wiley.

I would also like to thank my Kenyan associates who have helped me during my time in Kenya (the Achoka family, Titus Imboma, Onesmus Kioko, Ronald Mulwa, Alex Mutito, Nico Naliyana, and Vincent Otieno). Their help in the field was invaluable, but the “life-lessons” they have helped me learn (especially patience!), I will keep with me forever. Finally, I would like to thank advisors, professors, and colleagues at other institutions, particularly those from Southampton College and University of New Orleans, who helped to develop my knowledge and passion in biology before arriving at USF.
ACKNOWLEDGMENTS

I gratefully acknowledge several funding sources, which have provided considerable support throughout my dissertation research. I would like to thank the National Science Foundation (Doctoral Dissertation Improvement Grant), USF Graduate School (Challenge Grant), Sigma Xi (Grants-in-Aid of Research), Society for Integrative and Comparative Biology (Grants-in-Aid of Research), American Ornithological Society, and USF (Library Scholarship) for monies that supported my research in different capacities over the last five years. Also, I would like to thank Dr. Lynn Martin for generously supporting me with an RA during my whole Ph.D. career (via USF start-up and NSF IOS0920475); this support has allowed me the freedom to conduct extended field seasons in Kenya.

Additionally, I would like to acknowledge the many areas of business in Kenya that have allowed me to conduct my research on their premises. This includes (but is not limited to) the National Cereal and Produce Board of Kenya, KCC Nakuru, Petrozen, Total, and Oil Lybia petrol stations, and Timsales Timber yard. The permission of the many hotels that have allowed me to keep birds at the hotel is also greatly appreciated.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>iii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>iv</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>v</td>
</tr>
<tr>
<td>Abstract</td>
<td></td>
</tr>
<tr>
<td>Chapter One: Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Chapter Two: Exploratory behavior and stressor hyper-responsiveness facilitate range expansion of an introduced songbird</td>
<td>6</td>
</tr>
<tr>
<td>Abstract</td>
<td>6</td>
</tr>
<tr>
<td>Introduction</td>
<td>7</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>9</td>
</tr>
<tr>
<td>Study population</td>
<td>9</td>
</tr>
<tr>
<td>Exploratory behavior</td>
<td>10</td>
</tr>
<tr>
<td>Corticosterone response</td>
<td>11</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>12</td>
</tr>
<tr>
<td>Results</td>
<td>13</td>
</tr>
<tr>
<td>Exploratory behavior</td>
<td>13</td>
</tr>
<tr>
<td>Corticosterone release</td>
<td>14</td>
</tr>
<tr>
<td>Discussion</td>
<td>14</td>
</tr>
<tr>
<td>Exploratory behavior</td>
<td>14</td>
</tr>
<tr>
<td>Corticosterone response to stressors</td>
<td>15</td>
</tr>
<tr>
<td>Conclusions</td>
<td>18</td>
</tr>
<tr>
<td>Funding</td>
<td>19</td>
</tr>
<tr>
<td>Chapter Three: Stress hormone receptors change as range expansion progresses in house sparrows</td>
<td>31</td>
</tr>
<tr>
<td>Abstract</td>
<td>31</td>
</tr>
<tr>
<td>Introduction</td>
<td>32</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>34</td>
</tr>
<tr>
<td>Sampling</td>
<td>34</td>
</tr>
<tr>
<td>Sample storage</td>
<td>34</td>
</tr>
<tr>
<td>MR and GR gene expression</td>
<td>35</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>36</td>
</tr>
<tr>
<td>Results</td>
<td>36</td>
</tr>
<tr>
<td>Gene expression by DFM</td>
<td>36</td>
</tr>
<tr>
<td>Correlations between MR and GR</td>
<td>36</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

Table 2.1: Factors included in the generalized linear models determining the influences of exploratory behavior and corticosterone release in Kenyan house sparrows along a range expansion..................................................20

Table 2.2: PCA loadings for exploratory behavior in a novel environment (PC1) in 98 house sparrows from eight cities across a range expansion in Kenya. .......................................................................................................................21

Table 2.3: Top five models resulting from the generalized linear mixed models predicting exploratory behavior and corticosterone release in Kenyan house sparrows across a range expansion..................................................22

Table 3.1: Number of individuals and distance from Mombasa (km) of each capture site ...........................................................................................................................................40

Table 3.2: Quantitative PCR (qPCR) primers and probes used for house sparrow-specific gene quantification .....................................................................................................................................41

Table 4.1: Kenyan cities where house sparrows were collected and screened for variation in DNA methylation ........................................................................................................56
LIST OF FIGURES

Figure 2.1: Map of Kenya .................................................................23
Figure 2.2: Layout of novel room used to assess exploration .........................24
Figure 2.3: Scatterplots of exploratory behavior versus distance from Mombasa ....25
Figure 2.4: Relative importance of variables averaged across the top models predicting variation in exploration (a) and GC release (b) in Kenyan house sparrows undergoing a range expansion .........................................26
Figure 2.5: Exploratory behavior in males versus females ................................27
Figure 2.6: Scatterplots of corticosterone release during the breeding season versus distance from Mombasa ...........................................................................28
Figure 2.7: Scatterplots of corticosterone release during molt versus distance from Mombasa ..................................................................................................................29
Figure 2.8: Corticosterone release in urban versus rural house sparrows ...........30
Figure 3.1: Scatterplots of glucocorticoid receptor expression versus distance from Mombasa ..................................................................................................................42
Figure 3.2: Scatterplots of MR versus GR .....................................................43
Figure 4.1: Scatterplots of genetic diversity (H_o and F_is) versus epigenetic diversity (h) ..............................................................................................................................57
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔCORT</td>
<td>corticosterone release (difference between stress response and baseline)</td>
</tr>
<tr>
<td>AICc</td>
<td>Akaike Information Criteria</td>
</tr>
<tr>
<td>DFM</td>
<td>distance from Mombasa, Kenya</td>
</tr>
<tr>
<td>GCs</td>
<td>glucocorticoids</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>HPA</td>
<td>hippocampal pituitary adrenal axis</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
</tbody>
</table>
ABSTRACT

Introduced species cause both considerable ecological and economic damage every year. However, not much is known about how certain species are able to establish and spread beyond the site of initial introduction, whereas others do not. Species undergoing range expansion following an introduction may prove to be a valuable resource to invasion biology, but may also be informative in light of species’ responses to changing environments (i.e. global climate change). Here, I took advantage of an ongoing range expansion of an introduced vertebrate species. House sparrows (*Passer domesticus*) were introduced to Mombasa, Kenya in the 1950s and have subsequently expanded their range northwest-ward and now occupy most major cities in Kenya. By comparing older, established populations (i.e. those in Mombasa) with more recently colonized populations at the range edge, it might be possible to determine some of the mechanisms that underlie range expansion in some species and/or populations. In Chapter 1, the background and ideas that motivated the rest of the dissertation is summarized. In Chapter 2, I studied how exploration and glucocorticoids (a hormone released in response to stressors) changed throughout the range expansion. Exploration was greater at the range edge, which is likely to ensure greater discovery of novel resources. Glucocorticoids released in response to restraint were also highest at the range edge, which might facilitate resolution of stressors in unpredictable environments. However, chronically elevated levels of glucocorticoids are often considered maladaptive, unless an individual can
appropriately cope with them. Therefore, in Chapter 3, I characterized glucocorticoid receptors (i.e. mineralocorticoid receptor (MR) and glucocorticoid receptor (GR)) in the hippocampus, an area responsible for negative feedback of glucocorticoids as well as induction of behavioral and physiological response to stressors. I found that MR density was lower relative to GR density at the range edge compared to the site of introduction (Mombasa). I speculate this pattern is a mechanism to resolve the elevated levels of glucocorticoids at the range edge. Taken together, these results indicate that individuals at the range edge have a strong glucocorticoid response to stressors to induce a rapid, strong response to resolve stressors. Subsequently, in Chapter 4, I examined the potential mechanisms of phenotypic change among Kenyan house sparrows. Typically, following an introduction event, genetic diversity undergoes a bottleneck and is greatly reduced compared to the source population; as such, genetic evolution as the main driver of changing phenotypes throughout the range expansion is unlikely. We therefore hypothesized that epigenetic mechanisms (e.g. DNA methylation) may compensate for the expected reduced genetic diversity following an introduction. Although there was no pattern of epigenetic variation among cities (i.e. variation did not increase nor decrease further from the site of introduction), epigenetic variation increased as genetic inbreeding increased (a sign of reduced genetic diversity and bottlenecks), suggesting epigenetic modifications may compensate for reduced genetic diversity following an introduction event. Overall, patterns of phenotypic variation emerged dependent on age of the population- these patterns may prove to be important in other vertebrate range expansions as well. Surprisingly, epigenetic diversity did not correlate with phenotypic variation among populations; however, within-individual studies may reveal epigenotypes are
related to certain behavioral or physiological phenotypes. In the future, studies should be
designed to address how phenotypic differences arise despite relatively low genetic
diversity and overall high genetic admixture among individuals. In Kenyan house
sparrows, maintenance of high levels of flexibility and differential developmental
influences may be important factors that lead to varying phenotypes dependent on time
since colonization.
CHAPTER ONE: GENERAL INTRODUCTION

Introduced species are the second largest threat to global biodiversity. In the face of ecosystem impacts (Bakker and Wilson 2004), economic costs (Mack et al. 2000), and the likelihood of growing commerce increasing the threat of further introductions (Levine and D'Antonio 2003), invasive species research has grown considerably. However, studying invasive species, particularly during range expansion, in an ecological or evolutionary context would lend additional insight to the general spatial structure of species interactions, allopatric speciation, and response to environmental stressors and changes, such as global climate change (Holt 2003).

During a range expansion, individuals at the edge of a range potentially experience environments different from their native and/or previously introduced habitats (e.g. due to unfamiliar surroundings or density differences of conspecifics among sites). Due to contrasting selection pressures, individuals at different stages of a range expansion might demonstrate age of population-dependent traits. This would be particularly prominent if a trait imposed a cost only under certain conditions. For instance, in western bluebirds, selection pressures at different stages of colonization have led to different behavioral phenotypes: in males, aggressiveness increased competitive ability with conspecifics and therefore garnered a selective advantage in novel environments. However, as populations age, selection led to less aggressive, more philopatric males who exhibited greater parental care, which increased offspring survival.
Given the context-dependent advantages of either phenotype depending on the environment, it is clear that variable traits will be observed throughout a range expansion, although how these phenotypes arise is unknown and little studied.

Behavioral variation is important in establishment success post-introduction (Holway and Suarez 1999; Sol et al. 2002; Rehage and Sih 2004; Cote and Clobert 2007; Duckworth and Badyaev 2007; Duckworth 2008; Cote et al. 2010; Cote et al. 2010; Wright et al. 2010; Fogarty et al. 2011). However, how behaviors change throughout a range expansion and how selection acts to modify these behaviors is not as well understood. Within a range expansion, one behavior that is likely important in determining successful expansion is exploration, which tends to be high in invasive species compared to native conspecifics (Rehage et al. 2005; Cote et al. 2010; Russell et al. 2010). As with aggression, exploration may be differentially selected for at opposing ends of a range because in a novel environment, exploration of unfamiliar habitats and objects is required to gain information about potential resources and stressors. But, if exploration is associated with increased exposure to toxins and/or predators, or if the individuals who seek novelty are out-competed by individuals procuring known resources, exploration could be maladaptive in certain environments.

To cope, individuals respond to their environment both behaviorally and physiologically. One physiological system, the hypothalamic-pituitary-adrenal (HPA) axis, which releases glucocorticoids (GCs), is particularly important in helping organisms recognize and cope with harsh or stressful conditions. Though a strong, sustained GC release is sometimes maladaptive (McEwen and Wingfield 2003; Romero et al. 2009),
GCs promote the detection of possible threats, enhance memory (Packard and Williams 1995), and induce behaviors necessary to cope with a stressor (e.g. avoidance) (Orchinik 1998; Koolhaas et al. 1999). Enhanced detection, resolution, and memory of threats make the ability to increase GCs rapidly and strongly in an unpredictable environment adaptive (Love and Williams 2008).

However, adaptive value is expected only if individuals have the mechanisms (e.g. GC receptors) to respond appropriately to and cope with GCs. There are two main receptors for GCs, both found in high density in the hippocampus: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). MR has a higher affinity than GR for GCs and primarily regulates basal variation of the hormone (e.g. circadian and seasonal fluctuations in gluconeogenesis). GR, on the other hand, becomes bound and activated when GCs are elevated. Actions of GR restore homeostasis, induce memory formation, and subsequently stimulate the down-regulation of GCs (de Kloet 1991). An appropriate balance of MR and GR is necessary for proper functioning of the HPA axis and to stimulate appropriate behaviors and contextually organize memories; importantly, however, alterations to density can occur in response to environmental stimuli (de Kloet et al. 1998).

Many phenotypic changes are dependent on the environment experienced (both current and historic) and changes within a population as it transitions from new to colonized have been observed in just a few generations (Duckworth and Badyaev 2007; Gunnarsson et al. 2012). This rate of change cannot be explained by genetic evolution alone, especially in populations expanding from a site of invasion, where the introduction of few individuals frequently results in a bottleneck, limiting genetic diversity. An
additional source of variation is epigenetic modifications of the genome. Epigenetic modifications can alter gene expression and therefore phenotypes, without altering the DNA sequence (Richards 2006). There are several epigenetic mechanisms, including remodeling of chromatin and deacetylation of histones, but the most commonly studied is DNA methylation. Methylation involves the addition of a methyl group to a cytosine that is immediately followed by a guanine (Bossdorf et al. 2008). Epigenetic marks are stably inherited across generations (Jablonska and Raz 2009; Verhoeven et al. 2010) or generated de novo in response to an environment (Herrera et al. 2012). Epigenetic marks can be induced or removed in response to environmental cues throughout the lifetime of an individual (Angers et al. 2010; Verhoeven et al. 2010) and mediate some environmentally induced phenotypic variation (Dolinoj et al. 2007; Kucharski et al. 2008; Vogt et al. 2008; Angers et al. 2010; Bossdorf et al. 2010; Gao et al. 2010; Richards et al. 2010; Zhang et al. 2013). Changes in methylation not only influence mean trait values, but also the plasticity of certain traits (Bossdorf et al. 2010). Increased plasticity (i.e. the ability of an individual to adjust to environmental conditions) indicates that epigenetic mechanisms may mediate changes on a finer timescale than genomic evolution can; therefore, epigenetic processes, such as DNA methylation, may contribute to the success of an introduced population in novel habitats, even if genetic diversity is low (Pérez et al. 2006; Rando and Verstrepen 2007; Herrera et al. 2012; Richards et al. 2012; Schrey et al. 2012; Zhang et al. 2013).

Here, I document how certain behaviors (specifically, exploration) and physiological responses (GC response to stressors and receptor density in the hippocampus) were different at the edge of a range expansion of house sparrows (Passer
domesticus) in Kenya. House sparrows are one of the world’s most broadly distributed species and were introduced to Mombasa, Kenya in the 1950s (Anderson 2006). Since then, house sparrows have spread north-westward across the country arriving at the Kenyan-Ugandan border within the last few years. These sparrows make a particularly interesting system to study the effects of range expansion as the expansion history is relatively well known; additionally, the genetic structure of Kenyan house sparrows is relatively admixed (Schrey et al. in review), but with reduced genetic diversity compared to other house sparrows (Schrey et al. 2012). Due to the genetic constraints imposed on this population, I also report the epigenetic characteristics as a potential source of phenotypic variation among Kenyan house sparrows.
CHAPTER TWO: EXPLORATORY BEHAVIOR AND STRESSOR HYPER-
RESPONSIVENESS FACILITATE RANGE EXPANSION OF AN INTRODUCED
SONGBIRD¹

Abstract

Global anthropogenic changes are occurring at an unprecedented rate; one change, human-facilitated introduction of species outside their native range, has had significant ecological and economic impacts. Surprisingly, what traits facilitate range expansions post-introduction is relatively unknown. This information could help predict future expansions of introduced species as well as native species shifting their ranges as climate conditions change. Here, we asked whether specific behavioral and physiological traits were important in the ongoing expansion of house sparrows (*Passer domesticus*) across Kenya. We predicted that birds at the site of initial introduction (Mombasa, introduced ~1950) would behave and regulate corticosterone, a stress hormone, differently than birds at the range edge (Kakamega, ~885 km from Mombasa; colonized within the last 5 years). Specifically, we predicted greater exploratory behavior and stronger corticosterone response to stressors in birds at the range edge, which may facilitate the identification, resolution, and memory of stressors. Indeed, we found that distance from

¹ Portions of these results have been previously published (Liebl and Martin, 2012) and are utilized with permission of the publisher (Appendix A). Andrea L. Liebl and Lynn B. Martin designed the research and contributed analytic tools. The research was performed and analyzed by Andrea L. Liebl.
Mombasa (a proxy for population age) was a strong predictor of both exploratory behavior and corticosterone release in response to restraint (but only while birds were breeding). These results suggest that certain behavioral and neuroendocrine traits may influence the ability of species to colonize novel habitats.

Introduction

The introduction of species outside their native range is currently one of the largest threats to global biodiversity (Sala et al. 2000). Of the four stages of an invasion (i.e. introduction, colonization, establishment, and range expansion (Vermeij 1996; Cassey et al. 2004)), range expansion is arguably the most important because it is typically the stage in which species have the largest economic and ecological impacts. Due to the unethical nature of experimental introductions and the rarity of natural range expansions, they remain under-studied, especially in vertebrates. However, studying invasive species during a range expansion in an ecological or evolutionary context could lend great insight not only to invasion biology, but also to the spatial structure of species interactions, mechanisms of allopatric speciation, and population responses to environmental stressors, such as global climate change (Holt 2003).

For populations to expand, individuals must possess particular traits to allow them to exploit the novel conditions they face in new areas; many behavioral traits (e.g. boldness, aggression, response to novelty) may be especially important in novel environments (Holway and Suarez 1999; Sol et al. 2002; Rehage and Sih 2004; Martin and Fitzgerald 2005; Cote and Clobert 2007; Duckworth and Badyaev 2007; Duckworth 2008; Cote et al. 2010; Cote et al. 2010; Wright et al. 2010; Fogarty et al. 2011).
Different selection pressures along a range expansion could select for different levels of these traits between the site of introduction and the edge of the range. For instance, male mountain bluebirds (*Sialia currucoides*) are significantly more aggressive towards conspecifics in novel environments; however, as populations age, selection favors less aggressive, more philopatric males exhibiting greater parental care, which increases offspring survival (Duckworth and Badyaev 2007; Duckworth 2008). Exploratory behavior is also likely to be an important trait mediating range expansion, as exploration facilitates the identification of novel resources (Cole and Quinn 2011), as well as potential stressors (e.g. predators, challenging microclimates). However, when familiar resources are available, exploration might have a reduced benefit and be lost through genetic drift (Lawson Handley et al. 2011); further, in these areas, exploration might increase the likelihood of exposure to toxins and predators, waste time that could be devoted to other activities (Greenberg and Mettke-Hofmann 2001), and increase the likelihood of being out-competed by individuals procuring known resources. Indeed, exploration tends to be stronger in invasive species and invading populations (Rehage et al. 2005; Cote et al. 2010; Russell et al. 2010) compared to native species and populations.

In unfamiliar environments, where the necessity for exploration is increased, stressors are likely also less predictable and potentially more frequent. Glucocorticoids (GCs), hormones released by the hypothalamic-pituitary-adrenal axis in response to stressors, help individuals cope with and resolve stressors (Wingfield et al. 1998). Thus, the release of GCs in response to stressors is also apt to be stronger at range edges. Although data indicate that introduced populations are more exploratory than native ones
(Rehage et al. 2005; Cote et al. 2010; Russell et al. 2010) and GCs may play a role in population viability after an introduction (Martin et al. 2005), it remains untested whether increased exploration and altered stress hormone regulation facilitate range expansions.

Here, we tested whether exploratory behavior and corticosterone (the main avian GC) response to stressors are more pronounced at the edge of a range expansion of house sparrows (*Passer domesticus*) in Kenya. House sparrows are one of the world’s most broadly distributed species and were introduced to Mombasa, Kenya in the 1950s (Anderson 2006). Since then, house sparrows have spread north-westward across the country arriving at the Ugandan border within the last few years (Fig. 1). Using house sparrows collected from eight cities differing in time since colonization in Kenya, we measured exploratory behavior in a novel environment. We also measured corticosterone response to restraint stress along this range during both the breeding and non-breeding seasons.

**Materials and Methods**

**Study population**

Wild adult house sparrows (*Passer domesticus*) were caught in mist nets from eight cities across Kenya during the breeding season (March-June) of 2011 (Table 2.1; Fig. 2.1). To test the effect of life-history stage on corticosterone response (Romero 2002), additional house sparrows were caught from six of the eight cities in July, 2010 when Kenyan birds were molting (Table 2.1). House sparrows were introduced to Mombasa, Kenya around 1950. Although the year of house sparrow arrival to most other Kenyan cities is unknown, they likely arrived in Nairobi sometime during the late 1980s
or early 1990s (Lewis and Pomeroy 1989), and to cities north and west of Nairobi sometime after 2000 (National Museums of Kenya, unpublished data). Further, genetic analyses indicate that house sparrows expanded west from Mombasa along Mombasa highway (i.e. from Mombasa to Voi, to Nairobi, to Nakuru, to Kakamega) with a secondary expansion north from Nairobi (i.e. from Nairobi to Nyeri, to Isiolo; Schrey et al. in review). Therefore, we use distance from Mombasa (DFM) as a proxy for time since colonization (most recently colonized cities are furthest from Mombasa). At capture, birds were banded with a numbered aluminum band and color bands and wing cord (mm), tarsus (mm), and mass (g) were measured before being handled as described below. Individual condition was determined using the residuals of a linear regression of mass against tarsus. All experimental procedures were approved by the University of South Florida’s IACUC committee (W3877) and the Kenyan Ministry of Science and Technology.

Exploratory behavior

Between 8 and 18 adults were caught from each city (n= 98; Table 2.1). After capture, birds were brought into captivity and singly housed in 35.6 x 40.6 x 44.5 cm cages in ambient conditions. Birds were given ad libitum access to food (mixture of millet, red millet, sorghum, and rice) and water for one week during which time other behavioral measures were made for a separate study. Immediately following exploratory measurements, birds were released. Exploratory behavior was measured similarly to previously published methods (Verbeek et al. 1994; Dingemanse et al. 2002; Minderman et al. 2011; Mutzel et al. 2011). Briefly, after one hour without access to food, birds were
individually placed into a novel environment: a 2.74 m x 2.13 m tent containing ten novel items (table, cooking spoon, tent poles, broom, mop handle, antennae, stool, nest box, rope, and a bucket) and seams sufficient for perching (Fig. 2.2). Birds were given 10 sec to acclimate, then observed for 5 min by two individuals (and averaged for each variable). The proportion of the tent explored (measured in quarters, i.e. 25%, 50%, 75%, or 100%), the number of hops (when the individual changed location, not just direction), and the number of novel perches used were recorded as measures of exploratory behavior; additionally, the presence of stereotyped behaviors (e.g. patterns of repetitive movement) was assessed. All behavioral indices were collapsed into a single exploration score using a Principal Components Analysis (PCA; using correlation matrix and varimax rotation). PC1 was the only factor that met the Kaiser criterion (Eigenvalue >1), and therefore was the only factor used in analysis; PC1 accounted for 36% of the variation and varied positively with all four behavioral variables (Table 2.2).

Corticosterone response

During the breeding season, 10-13 house sparrows (n= 88, Table 2.1) were collected from each of the eight cities; another 5-11 house sparrows (n= 58; Table 2.1) were collected from six of the eight cities during molt. To measure corticosterone concentrations, blood (~25 μl) was taken from the brachial vein within 3 min of capture; birds were then placed in a cloth bag for 30 min to elicit a corticosterone response, and bled again. Blood was centrifuged and plasma was extracted and frozen in liquid nitrogen until corticosterone levels could be measured using a commercially available EIA kit (Assay Designs; average detection limit of 27 pg; validated elsewhere (Breuner et
Samples were randomly distributed among eight plates; intra-plate variation was less than 10% for each plate, whereas average inter-plate variation was 8%. Corticosterone response (ΔCORT) was calculated by subtracting baseline values of corticosterone from elevated levels taken after 30 min of restraint. Different individuals from each site were used for this part of the study because i) repeated bleeding could have affected behavior (van Oers and Carere 2007), and ii) experiments were not designed to address the mechanism of action of corticosterone on exploratory behavior.

**Statistical analysis**

ΔCORT was ln-transformed to achieve normality. General linear models (GLMs) were used to determine whether population age (i.e. DFM; km) predicted exploratory behavior and ln-corrected ΔCORT responses; as corticosterone is regulated differently during breeding compared to molting (Romero 2002), corticosterone data were analyzed separately by season. When GLMs indicated significant effects of DFM on measured traits, we then used a model selection approach (using general linear mixed models (GLMM)), to determine whether DFM was a better predictor of dependent variables than other factors known to influence behavior or corticosterone (see below). In addition to DFM, we used the degree of urbanization around the catching site (microhabitat), altitude (m), house sparrow density (per km²), and individual condition and sex (Table 2.1) as fixed factors in GLMMs and each individual nested within city was used as a random factor. DFM and altitude were determined using a GPS device (Garmin 60 CSx); microhabitat, shown to affect corticosterone regulation in other studies (Partecke et al.
was assessed as urban or non-urban by the proportion of pavement surrounding the netting site (within ~50 m) and the amount of vehicular and human traffic through the area; and house sparrow density was determined at the time of data collection by averaging point count estimates (two observers, 5 min fixed-radius (50 m) distributed throughout each city (8-15 per city, depending on city size; Martin et al., in review). Backwards model selection was conducted based on corrected Akaike Information Criteria (AICc) scores; each single factor was used as well as interactions between DFM and microhabitat, DFM and condition, DFM and sex, condition and house sparrow density, and microhabitat and density; we chose these interactions because microhabitat, condition, and sex may vary by DFM (dependent on the dispersal mechanism) and density might influence individual condition or be influenced by microhabitat. Using AICc scores, the top five models were averaged to determine the relative importance of each variable (Bolker et al. 2009). R 2.14.0 was used for all statistical analyses and GraphPad Prism 5 was used to make the figures.

Results

Exploratory behavior

As predicted, during the breeding season, individuals at the range edge were most exploratory, whereas individuals from the site of initial introduction (Mombasa) were least exploratory (F_{1,97} = 7.937, p= 0.006; Fig. 2.3). Model selection indicated that DFM was the best predictor of exploratory behavior (estimate= 0.0010 +/- 0.0004; Table 2.3; Fig. 2.4a). Additionally, sex was an important predictor of exploratory behavior.
(estimate= -0.5034 +/- 0.3330; Table 2.4): males (n= 50) were significantly more exploratory than females (n= 48; t= 2.50, p= 0.014; Fig. 2.5).

*Corticosterone release*

During the breeding season, individuals from the most recently colonized cities (i.e. those furthest from Mombasa) released significantly more corticosterone in response to a restraint stressor than those from the longer established cities ($F_{1,86} = 2.131$, $p= 0.0359$; Fig. 2.6); however, when molting, no such relationship existed ($F_{1,55} = 0.985$, $p= 0.33$; Fig. 2.7). Interestingly, differences among populations were driven by corticosterone levels in response to a stressor during breeding, as baseline levels of corticosterone did not differ among cities (breeding: $F_{7,89} = 0.974$, $p= 0.46$; molting: $F_{1,47} = 1.514$, $p= 0.140$). Model selection revealed that DFM was one of the best predictors of $\Delta$CORT during breeding (estimate= 0.00044 +/- 0.0003; Table 2.1; Fig. 2.4b), however, the best model also included urbanization of the catching site (estimate= 0.2803 +/- 0.1309; Table 2.1; Fig. 2.4b). House sparrows caught from more urban areas released significantly less corticosterone than those caught from rural areas ($t= 3.67$, $p< 0.001$; Fig. 2.8). At the population level, no correlation was observed between exploration and corticosterone release ($p= 0.815$), although previous studies might predict such a relationship (Koolhaas et al. 1999).

**Discussion**

Behavioral and physiological differences exist among populations of a species that has colonized a novel environment within just the last 60 years. The observed
patterns are consistent with the hypothesis that these traits facilitated the range expansion. It is yet unclear whether phenotypic plasticity, genetic differentiation, or both underlie the patterns described here, but below we describe how one might discriminate between these possibilities and provide possible interpretations of our present data.

*Exploratory behavior*

Although decreased exploration might be protective or unnecessary (and therefore lost due to drift in the absence of reinforcing selection) in familiar habitats, increased exploration might be adaptive in novel environments such as those found at the edge of a range. Exploratory behavior would allow individuals to discover and procure novel resources in unfamiliar habitats as well as identify potential stressors when such information is less readily available (e.g. from conspecifics). Within the novel environment, males were significantly more exploratory than females. Both males and female house sparrows provision and care for chicks after hatch, but males typically locate and defend nesting sites before breeding; increased exploratory behavior in males could enhance the acquisition of quality nesting sites, increasing fitness. Although other studies have shown that males of other species colonize new territories before females (Duckworth and Badyaev 2007), it is unknown whether Kenyan house sparrows disperse in a sex-dependent manner.

Although exploratory behavior was tested in an artificial environment, without any food reward, we feel our paradigm was representative of exploratory behavior in the wild. In European starlings (*Sturnus vulgaris*), the amount of a novel environment (one similar to that used in this experiment) explored, but not the speed of exploration, tended
to be correlated with the maximum home range size of that individual (Minderman et al. 2010). Further, although a few individuals displayed frantic movements in the tent, possibly indicative of increased stress and in search of an escape, most calmly hopped through the novel environment pecking at things on the ground or even preening (A.L., pers obs).

_Corticosterone responses to stressors_

Corticosterone release also varied among populations with range-edge populations releasing the most corticosterone, although only during the breeding season. Elevated GC responses at the edge of the range may increase vigilance, aiding in the detection of stressors in novel environments, which may offset the costs of exploration, such as increased exposure to predators and parasites. Further, elevated GC responses may also facilitate the consolidation or formation of memories for novel resources and stressors alike (de Kloet 1991). In other words, strong, rapid elevations of corticosterone in response to stressors might allow individuals at range edges to mitigate and/or remember stressors better in environments where stressors are potentially less predictable, less familiar, and/or more numerous.

Corticosterone release was also related to the degree of urbanization close to the catching site. Urbanization affects corticosterone regulation in other species as well (Partecke et al. 2006; Fokidis et al. 2009; Zhang et al. 2011) and a damped corticosterone response in urban areas may reflect habituation to stressors such as increased noise and human disturbance. Here we used ΔCORT as an index of corticosterone regulation, rather than absolute levels of the hormone to control for individual variation in
corticosterone regulation mechanisms (e.g. corticosterone receptors, corticosterone-binding-globulins). Other studies have suggested that these factors may be controlled by baseline levels of corticosterone (de Kloet et al. 1998; Müller et al. 2009), which did not vary among populations at either time of year. Although this study was not designed to elucidate the hormonal mechanisms of exploratory behavior, previous studies indicate that a relationship between corticosterone release and exploratory behavior might exist; however, no such within-population relationship was observed. A lack of a relationship may be a result of using different birds in each population for hormonal and exploratory measurements, however recent papers have argued this relationship may be more tenuous than previously thought (Coppens et al. 2010; Koolhaas et al. 2010).

In the Kenyan house sparrow colonization, low genetic diversity (Schrey et al. 2011) and the unlikelihood of an influx of genetic variation from other areas where house sparrows occur (Schrey et al., in review) make it somewhat surprising that such extensive phenotypic distinction is observed at all among populations. In this and other examples (Duckworth and Badyaev 2007; Gunnarsson et al. 2012), the rapid change of trait distributions along a range expansion suggest that phenotypic plasticity or rapid evolution allowed the differentiation among populations. However, how these patterns arise is unknown. Interestingly, ΔCORT differences among populations were only significant when individuals were breeding, a time when mothers might deposit hormones to the yolk of her developing offspring. In other taxa, maternal transfer of corticosterone to eggs has many strong physiological (Bakker et al. 2001) and behavioral effects (Freire et al. 2006), including enhancement of the corticosterone response to stressors (Bakker et al. 2001). Another non-genetic parental effect could be behavioral influences: if exploratory
behavior is elevated at the range edge, offspring provisioning might be reduced if parents take longer to find food; such absence cues might be used as an indication of environmental quality, influencing offspring phenotype (Love and Williams 2008). In rats, reduced maternal care causes an enhanced corticosterone response (Meaney 2001), increased vigilance (Meaney 2001), and improved hippocampal-dependent learning under stressful conditions due to epigenetic alterations (methylation) of the glucocorticoid receptor promoter in the hippocampus (Weaver et al. 2004). Although it is unknown if similar mechanisms occur in birds, or what the specific developmental window might be, other studies indicate that parental behavior during the juvenile period might also be an important time in the development of offspring behavior and corticosterone regulation in birds (Cyr and LM 2007; Love and Williams 2008; Banerjee et al. 2011).

**Conclusions**

Kenyan house sparrows at the edge of a range expansion were significantly more exploratory in a novel environment and released significantly more corticosterone during the breeding season compared to house sparrows at the site of initial introduction; these patterns suggest that these traits may have influenced the Kenyan colonization. Ongoing studies are investigating i) whether and how exploratory behavior and corticosterone release are related to fitness among populations, and ii) how genetic, epigenetic, and maternal effects influence phenotypic diversity. We hope that our results inspire efforts to determine whether exploratory behavior and stress-coping mechanisms affect range expansions in other species; if so, parts of species’ ranges most likely to expand might be
revealed and pest control efforts adjusted accordingly. Likewise, exploration and
corticosterone release could prove important for species’ extinction risk, as low
exploratory behavior and weak stressor responsiveness might hinder populations’
adjustments to altered environments.

Funding

The National Science Foundation (IOS 0920475) and the University of South
Florida to LBM, and Society for Integrative Biology (Grant in Aid of Research) and
Sigma Xi (Grant in Aid of Research) to ALL.
Table 2.1. Factors included in the generalized linear models determining the influences of exploratory behavior and corticosterone release in Kenyan house sparrows along a range expansion.

<table>
<thead>
<tr>
<th>city</th>
<th>distancea</th>
<th>micro-habitatb</th>
<th>altitudec</th>
<th>densityd</th>
<th>behavior</th>
<th>corticosterone M/F (=breeding)</th>
<th>corticosterone M/F (=molting)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mombasa</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>81.19</td>
<td>7/10</td>
<td>5/7</td>
<td>10</td>
</tr>
<tr>
<td>Watamu</td>
<td>120</td>
<td>1</td>
<td>0</td>
<td>21.39</td>
<td>5/7</td>
<td>8/4</td>
<td>10</td>
</tr>
<tr>
<td>Voi</td>
<td>160</td>
<td>1</td>
<td>591</td>
<td>17.54</td>
<td>8/4</td>
<td>7/6</td>
<td>5</td>
</tr>
<tr>
<td>Nairobi</td>
<td>500</td>
<td>0</td>
<td>1690</td>
<td>45.41</td>
<td>7/5</td>
<td>6/7</td>
<td>11</td>
</tr>
<tr>
<td>Nakuru</td>
<td>630</td>
<td>0</td>
<td>1768</td>
<td>12.52</td>
<td>4/6</td>
<td>7/5</td>
<td>11</td>
</tr>
<tr>
<td>Nyeri</td>
<td>650</td>
<td>0</td>
<td>1945</td>
<td>15.21</td>
<td>4/4</td>
<td>8/5</td>
<td>N/A</td>
</tr>
<tr>
<td>Isiolo</td>
<td>755</td>
<td>0</td>
<td>1145</td>
<td>33.53</td>
<td>5/7</td>
<td>5/6</td>
<td>N/A</td>
</tr>
<tr>
<td>Kakamega</td>
<td>885</td>
<td>1</td>
<td>1530</td>
<td>12.46</td>
<td>11/7</td>
<td>6/5</td>
<td>11</td>
</tr>
</tbody>
</table>

a. Distance from Mombasa, the site of introduction; km  
b. Urban (1) or non-urban (0)  
c. Above sea level; m  
d. House sparrows per km² determined using point count estimates  
e. Male to female sex ratio
Table 2.2. PCA loadings for exploratory behavior in a novel environment (PC1) in 98 house sparrows from eight cities across a range expansion in Kenya. PC1 explained 36% of the variance.

<table>
<thead>
<tr>
<th>variable</th>
<th>PC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of hops</td>
<td>0.468</td>
</tr>
<tr>
<td>number of novel perches used</td>
<td>0.501</td>
</tr>
<tr>
<td>proportion of the tent explored</td>
<td>0.627</td>
</tr>
<tr>
<td>presence of stereotypic behavior</td>
<td>0.369</td>
</tr>
</tbody>
</table>
Table 2.3. Top five models resulting from the generalized linear mixed models predicting exploratory behavior and corticosterone release in Kenyan house sparrows across a range expansion. Distance from Mombasa (the site of introduction; distance), sex, condition, microhabitat (urban or non-urban), altitude, and house sparrow density were treated as fixed factors and individuals nested within city was treated as a random factor.

<table>
<thead>
<tr>
<th>factors</th>
<th>AICc</th>
<th>Δ AICc</th>
<th>K</th>
<th>log-likelihood</th>
<th>weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>exploratory behavior</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>distance+ sex</td>
<td>316.28</td>
<td></td>
<td>2</td>
<td>-151.68</td>
<td>0.52</td>
</tr>
<tr>
<td>distance+ sex+ distance x sex</td>
<td>317.49</td>
<td>1.22</td>
<td>3</td>
<td>-151.13</td>
<td>0.28</td>
</tr>
<tr>
<td>distance+ condition+ sex+ condition x sex</td>
<td>319.81</td>
<td>3.53</td>
<td>4</td>
<td>-151.10</td>
<td>0.09</td>
</tr>
<tr>
<td>distance</td>
<td>320.65</td>
<td>4.37</td>
<td>1</td>
<td>-155.00</td>
<td>0.06</td>
</tr>
<tr>
<td>sex</td>
<td>321.39</td>
<td>5.11</td>
<td>1</td>
<td>-155.37</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>corticosterone release</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>distance+ microhabitat</td>
<td>155.74</td>
<td></td>
<td>2</td>
<td>-71.35</td>
<td>0.33</td>
</tr>
<tr>
<td>microhabitat</td>
<td>156.35</td>
<td>0.62</td>
<td>1</td>
<td>-72.81</td>
<td>0.24</td>
</tr>
<tr>
<td>altitude+ distance+ microhabitat</td>
<td>157.21</td>
<td>1.47</td>
<td>3</td>
<td>-70.90</td>
<td>0.16</td>
</tr>
<tr>
<td>distance</td>
<td>157.56</td>
<td>1.82</td>
<td>1</td>
<td>-73.41</td>
<td>0.13</td>
</tr>
<tr>
<td>altitude+ distance+ microhabitat+ sex</td>
<td>157.68</td>
<td>1.94</td>
<td>4</td>
<td>-69.93</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Figure 2.1. Map of Kenya. House sparrows were introduced to Mombasa, Kenya in the 1950s and have subsequently spread across Kenya and into Eastern Africa. In this study, house sparrows were captured from eight cities (Mombasa, Malindi, Voi, Nairobi, Nakuru, Nyeri, Isiolo, and Kakamega) indicated by blue squares on the map. Distance from Mombasa (DFM; km) was used as a proxy of time since colonization, as cities furthest from Mombasa are likely the most recently colonized by house sparrows. Figure was adapted from the USA CIA’s open database.
Figure 2.2. Layout of novel room used to assess exploration. A 2.74 m x 2.13 m novel room with 10 novel perches ((A) broom, (B) broom pole, (C) upside-down stool, (D) rope, (E) tent poles, (F) antennae, (G) nest box, (H) cooking spoon, (I) table on side, and (J) bucket hanging from rope) and seams (K) for perching around the edge. The proportion of the room explored, the number of perches used, the number of hops, and the presence of repetitive, aggressive movement directed towards objects or tent walls were recorded for 5 min.
Figure 2.3. Scatterplots of exploratory behavior versus distance from Mombasa. Distance from Mombasa is related to exploratory behavior in Kenyan house sparrows (n= 98). House sparrows at the edge of the range expansion are more exploratory than birds from the site of introduction (~60 years since establishment). Distance from Mombasa was the best predictor of exploration of a novel habitat (F= 7.937, p= 0.006; estimate= 0.0010 +/- 0.0004). Exploratory behavior (PC score of combined exploratory variables) is averaged by city and regression bands are 95% confidence intervals.
Figure 2.4. Relative importance of variables averaged across the top models predicting variation in exploration (a) and GC release (b) in Kenyan house sparrows undergoing a range expansion.
Figure 2.5. Exploratory behavior in males versus females. Male Kenyan house sparrows (n=50) were more exploratory than females (n=48) (estimate= -0.5034 +/- 0.3330; t= 2.50, p= 0.014) in a novel environment; means +/- SEM are presented.
Figure 2.6. Scatterplots of corticosterone release during the breeding season *versus* distance from Mombasa. Corticosterone release is related to distance from Mombasa in Kenyan house sparrows only during the breeding season (n= 88). When breeding, house sparrows at the edge of a range expansion released more corticosterone, a stress hormone, in response to a stressor than birds from the site of introduction ($F_{1,86} = 2.131$, $p = 0.0359$); model selection indicated that distance from Mombasa was one of the most important predictors of variability in $\Delta$CORT. The average residuals of ln-corrected $\Delta$CORT and microhabitat for each population are plotted against distance from Mombasa with 95% confidence intervals.
Figure 2.7. Scatterplots of corticosterone release during molt versus distance from Mombasa. While molting (n= 56), no significant differences in ΔCORT among Kenyan house sparrow populations were found (F_{1,55} = 0.985, p= 0.33). The average ln-corrected ΔCORT for each population are plotted against distance from Mombasa.
Figure 2.8. Corticosterone release in urban versus rural house sparrows. House sparrows from more urban sites released significantly less corticosterone in response to 30 min restraint (estimate= 0.2803 +/- 0.1309; t= 3.67, p<0.001); means +/- SEM are presented.
CHAPTER THREE: STRESS HORMONE RECEPTORS CHANGE AS RANGE EXPANSION PROGRESSES IN HOUSE SPARROWS

Abstract

As ranges expand, individuals encounter different environments at the periphery than at the center of the range. Previously, we have shown that glucocorticoids (GCs) vary with range expansion: individuals at the range edge release more GCs in response to restraint. Here, we measured hippocampal mRNA expression of GC receptors (mineralocorticoid (MR) and glucocorticoid (GR)) in eight house sparrow (*Passer domesticus*) populations varying in age. We found that individuals closest to the range edge had the lowest expression of MR relative to GR; in all likelihood, this relationship was driven by a marginal reduction of MR mRNA at the range edge. Reduced MR (relative to GR) might allow enhanced GC binding to GR, the lower affinity receptor, which would enhance a rapid physiological and behavioral response to stressors. The insights gained from this study are not only enlightening to introduced species, but may also predict how certain species will react as their ranges shift due to anthropogenic changes.

---

2 Portions of these results have been previously published (Liebl and Martin, 2013) and are utilized with permission of the publisher (Appendix A). Andrea L. Liebl and Lynn B. Martin designed the research and contributed analytic tools. The research was performed and analyzed by Andrea L. Liebl.
Introduction

One of the largest threats to global biodiversity is the introduction and spread of non-native species (Sala et al. 2000). In novel habitats, individuals may face more unpredictable stressors (e.g. unknown/novel resources, predators/parasites); to cope with these stressors, vertebrates often release glucocorticoids (GCs; (Wingfield et al. 1998)). Significant variation in the regulation of and response to GCs exists, and how an individual physiologically and behaviorally responds to GCs dictates its fitness in certain contexts. GCs can enhance vigilance and memory consolidation (Packard and Williams 1995), and mediate behaviors necessary to survive stressors (e.g. avoidance (Koolhaas et al. 1999)), all of which may increase survival in unpredictable environments. Indeed, in a population undergoing range expansion, individuals at the range edge (where stressors and resources are potentially less known) released more GCs in response to restraint than those from more established areas (Liebl and Martin 2012).

Basal GCs, which respond to daily and seasonal fluctuations, are controlled by mineralocorticoid receptors (MR), whereas glucocorticoid receptors (GR) predominantly mediate physiological and behavioral changes necessary to restore homeostasis after a stressor has been encountered (de Kloet 1991). In the hippocampus, which plays a role in GC negative-feedback, MR and GR work in a coordinated and antagonistic fashion to mediate GCs (i.e. the MR/GR balance hypothesis (de Kloet 1991)). It has been suggested that coordinated fluctuations in MR and GR allow greater physiological flexibility in response to GCs (Evans and Arriza 1989). Also, when MR and GR are incubated together in vitro, they show enhanced binding to glucocorticoid response elements compared to when incubated individually; the composition of the two receptors
ultimately determines binding efficiency, with the greatest binding when concentrations of GR exceed MR (Trapp et al. 1994). These authors concluded that “the cooperativity of MR and GR in DNA binding suggests a direct interaction between these two receptors” and “when MR and GR are expressed in the same cell, their relative levels…will define which corticosterone receptor dimer [each homodimer versus the heterodimer]...is constituted [which] enables a more finely tuned regulation of corticosterone responsive genes” (Trapp et al. 1994). Greater density of GR relative to MR should therefore facilitate enhanced resolution of stressors. Also, in rodents (Patchev et al. 1994) and birds (Liebl et al. 2013), the combination of MR and GR, not the concentration of either alone, dictated phenotypic effects of GCs. Although measuring absolute hormone concentrations is informative, understanding whole systems, including receptors, might further illuminate how variation arises (Wingfield and Mukai 2009).

Although we know GCs change throughout range expansion (Liebl and Martin 2012), we know little about how hormone receptors respond; importantly, it is hormone-receptor-complexes (Wingfield and Mukai 2009) that initiate and mediate downstream effects of GCs. To address this point, we measured expression of hippocampal MR and GR mRNA throughout a range expansion. House sparrows (*Passer domesticus*) are expanding northwest from their most recent introduction site, Mombasa, Kenya (introduced ~1950 (Anderson 2006)). Individuals caught from areas near the range edge released more GCs in response to restraint than those from Mombasa. We predicted that mRNA expression of MR relative to GR would be lowest closest to the range edge to facilitate stressor resolution afforded by activated GR and rapid down-regulation of GCs after the stressor is resolved (de Kloet 1991).
Materials and Methods

Sampling

In February-May, 2011, house sparrows were caught from 8 cities in Kenya (Mombasa, Malindi, Voi, Nairobi, Nyeri, Nakuru, Isiolo, and Kakamega; Fig. 2.1). We use distance from Mombasa (DFM) as a proxy for time since colonization (most recently colonized cities are furthest from Mombasa (Liebl and Martin 2012; Schrey et al. in review)). Adult birds (n=6-13, median=9; Table 3.1) were caught from each city and individual sex, tarsus, and mass were recorded. Within 15 min of capture, birds were deeply anesthetized and decapitated. The hippocampus was removed immediately (by conservatively cutting around the anterior perimeter of the hippocampus and pulling from the forebrain (Liebl et al. 2013) with RNase-free tools) and stored in RNAlater (Qiagen). All procedures were approved by University of South Florida’s IACUC committee and the Kenyan Ministry of Science and Technology.

Sample storage

After collection, all hippocampi were stored in RNAlater (Qiagen) until they could be frozen. However, for some sites, samples were stored at room temperature (~27°C) for 12 weeks, whereas others were stored at room temperature for only 3 weeks; the sampling order was not conducted in a manner consistent with distance from Mombasa (i.e. we collected tissues from Nakuru (650 km from Mombasa) first, then Kakamega (885 km), then Mombasa, Watamu (120 km), Voi (160 km), Nairobi (500 km), Isiolo (755 km), and finally Nyeri (630 km)). Although Qiagen states that RNAlater
protects and stabilizes RNA expression patterns even after stored under a wide variety of conditions (including room temperature and freeze/thaw cycles), we performed a General Linear Model to confirm that the MR:GR ratio did not vary with the amount of time samples spent at room temperature ($F_{1,6}= 0.007; p= 0.937$).

**MR and GR gene expression**

RNA was extracted from the hippocampus ($\leq 30$ mg) using a rotor-stator homogenizer and an RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions; RNA concentration was determined using a spectrophotometer. cDNA was synthesized from up to 0.5 µg/µl total RNA with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) following manufacturer’s instructions; using a spectrophotometer, all cDNA was determined to be free of contamination (260/280 ratio = 1.8-2.1) and was diluted to ~150 ng/µl.

MR and GR gene expression were measured using qPCR (Applied Biosystems) with ~300 ng cDNA, MasterMix (Applied Biosystems), and multiplexed primers and probes specific for house sparrow MR and GR (Table 3.2; (Liebl et al. 2013)); ultrapure water was used as a negative control and a 4-step standard curve was made (300, 100, 30, and 11 ng/ul) using a homogenate of cDNA from three individuals from each city, randomly chosen. A housekeeping gene, 18S, was also measured to determine total RNA activity in the tissue, and both target genes (MR and GR) were adjusted to expression of 18S (Applied Biosystems). qPCR was run using the conditions: 50°C for 2 min, 95°C for 10 min, and then 40 cycles: 95°C for 15 sec and 60°C for 1 min.
**Statistical analysis**

Target genes were adjusted to 18S (by dividing expression values of the target gene by that of 18S, Applied Biosystems). To generate an MR:GR ratio (de Kloet 1991), adjusted gene expression of MR was divided by the adjusted gene expression of GR, which were then log-transformed to achieve normality. General linear models were used to analyze the effect of DFM on the ratio of MR:GR gene expression, as well as MR and GR alone. Additionally, Spearman Correlations were used to address within-individual covariation of MR and GR, with the expectation that high covariation would provide additional support for the use of the MR:GR ratio. Statistica 9.1 was used to perform all analyses, with α=0.05. Data are deposited in Dryad (Liebl and Martin 2013).

**Results**

**Gene expression by DFM**

The MR:GR gene expression ratio was significantly lower in individuals from populations closest to the edge of the range expansion (F <sub>1,76</sub> = 5.385, p = 0.023; Fig. 3.1a). Gene expression of MR alone was marginally lower in newer populations (F <sub>1,76</sub> = 2.860, p = 0.095; Fig. 3.1b), whereas GR was not explained by DFM (F <sub>1,76</sub> = 0.435, p = 0.5114; Fig. 3.1b).

**Correlations between MR and GR**

Individual expression of MR was positively correlated with expression of GR (r = 0.7311; p < 0.001; Fig. 3.2).
Discussion

*GC receptors throughout a range expansion*

GC regulation is important because GCs respond to internal and external environmental changes (Wingfield and Mukai 2009) allowing organisms to morphologically, physiologically, and behaviorally respond to and resolve stressors and perturbations. Both MR and GR regulate GC negative-feedback in the hippocampus (de Kloet 1991), but, physiologically, GR, the lower affinity of the two receptors (de Kloet 1991), is predominantly responsible for actions of a stress response. Bound GR stimulates changes (e.g. reduced metabolism, avoidance) that promote survival of a stressor (de Kloet 1991). Further, MR and GR heterodimers have greater binding capacity, and potential effect, when concentrations of GR are greater than MR (Trapp et al. 1994). Therefore, a greater density of GR relative to MR should facilitate a greater and/or more rapid resolution of encountered stressors; in novel habitats, a strong and rapid response is likely especially favorable.

Although elevated GCs at the range edge (Liebl and Martin 2012) may induce changes in receptor expression (de Kloet 1991; Hodgson et al. 2007), it is remarkable that such strong patterns of gene expression emerge with population age. Traditionally, it is thought that selection favors the persistence of conserved homeostatic systems, independent of environmental cues (Wingfield and Mukai 2009); this is particularly true of the physiological components responsible for receiving, processing, and signaling (internal and external) environmental information (Woods 2009), such as GC receptors. This view predicts that GC receptor density would be conserved throughout the range expansion, and throughout the lifetime of an individual, regardless of environmental
differences/changes. This is because changes in receptor density might alter physiological set-points, potentially leading to fitness consequences in unknown environments (Woods 2009). However, our data indicate that not only do GC receptors differ throughout a range expansion, but they vary in a manner consistent with the range expansion itself. This variation in GC responsiveness likely permits alterations in downstream physiological and behavioral events to best respond to changing and unpredictable environments (Martin et al. 2011).

Given the likely mechanism of expansion in this population (human mediated, (Schrey et al. *in review*)), it is unlikely that all individuals arriving to new habitats are pre-adapted to survive there. Rather, we hypothesize that variation exists among individuals arriving at the range edge (possibly exemplified by the relatively high MR:GR ratio in Kakamega, the youngest population), but only individuals with a low MR:GR ratio, or those with the flexibility to reduce it rapidly in response to the novel environment, will survive (as exemplified by the low MR:GR ratio in Isiolo, Nyeri, and Nakuru, populations <10 years old, and intermediate levels in Nairobi, <20 years old). Perpetuation of distinct phenotypes among populations might be a result of rapid evolution, developmental plasticity (early life experiences can shape GC receptor densities in the brain (Weaver et al. 2004; Banerjee et al. 2011)), or simply phenotypic flexibility responding to specific cues in the environment (Cheviron et al. 2008); further, the adaptive value of these phenotypes are as yet unknown. Ultimately, however, the current experiment was not designed to elucidate the mechanism(s) of change, nor the adaptive values of those changes. In the future, experiments discerning the roles of
selection, development, and adaptive plasticity (Ghalambor et al. 2007) in the GC receptor changes we describe here would be particularly interesting.

Conclusions

GCs have strong, but complex effects on fitness in wild animals (Breuner et al. 2008), however relatively little is known on what role GC receptors play. We argue that regulation of the signal is just as important as the signal itself. In a world where many environments are rapidly changing, often due to anthropogenic changes, information such as that presented here might be particularly informative in predicting population outcomes.

Funding

The National Science Foundation (LBM; IOS-0920475), University of South Florida (LBM), American Ornithologist’s Union (ALL), Society for Integrative Biology (ALL), and Sigma Xi (ALL).
Table 3.1. Number of individuals and distance from Mombasa (km) of each capture site. mRNA expression of MR and GR were measured in house sparrows captured from eight cities across Kenya. Numbers of individuals (n) and distance from Mombasa (km) of each capture site are presented here; distance from Mombasa significantly predicted changes in the mRNA expression of MR relative to GR.

<table>
<thead>
<tr>
<th>city</th>
<th>n</th>
<th>km from Mombasa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mombasa</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Malindi</td>
<td>8</td>
<td>120</td>
</tr>
<tr>
<td>Voi</td>
<td>13</td>
<td>160</td>
</tr>
<tr>
<td>Nairobi</td>
<td>6</td>
<td>500</td>
</tr>
<tr>
<td>Nyeri</td>
<td>11</td>
<td>630</td>
</tr>
<tr>
<td>Nakuru</td>
<td>10</td>
<td>650</td>
</tr>
<tr>
<td>Isiolo</td>
<td>10</td>
<td>755</td>
</tr>
<tr>
<td>Kakamega</td>
<td>9</td>
<td>885</td>
</tr>
</tbody>
</table>
Table 3.2. Quantitative PCR (qPCR) primers and probes used for house sparrow-specific gene quantification (Liebl et al. 2013).

<table>
<thead>
<tr>
<th>Genbank #</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR 1174545</td>
<td>CTGTTAAGATCCTTGAAAG</td>
<td>GGTTCAGGATGGAAAGCAGG</td>
<td>FAM-CAGGATACGACAGCTC</td>
</tr>
<tr>
<td></td>
<td>CATTGAG</td>
<td>TA</td>
<td></td>
</tr>
<tr>
<td>GR 1285164</td>
<td>ACCTCTCTGGCAAGCTGC</td>
<td>GTTGTGGATGGAGAAGCTAGCT</td>
<td>VIC-CTCTAATGGCTATTGAAGC</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>TACAT</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1. Scatterplots of glucocorticoid receptor expression versus distance from Mombasa. Expression of stress steroid receptor mRNA varies along a range expansion in introduced Kenyan house sparrows. (a) House sparrows at the edge of a range (right-most points) had significantly lower hippocampal MR:GR gene expression ratios ($F_{1,76}=5.385$, $p=0.023$) than house sparrows at the site of introduction (left-most point). (b) House sparrows at the edge of the range had marginally lower levels of MR (▲; $F_{1,76}=2.860$, $p=0.095$), but no differences in GR (■; $F_{1,76}=0.435$, $p=0.5114$) than those from the site of introduction. Average log-corrected values are plotted against distance from Mombasa.
Figure 3.2. Scatterplots of MR versus GR. Gene expression of MR was positively correlated with that of GR (r=0.731; p<0.001).
CHAPTER FOUR: PATTERNS OF DNA METHYLATION THROUGHOUT A RANGE EXPANSION OF AN INTRODUCED SONGBIRD

Abstract

The spread of invasive species presents a genetic paradox: how do individuals overcome the genetic barriers associated with introductions (e.g. bottlenecks, founder effects) to become adapted to their new environment? In addition to genetic diversity, epigenetic variation also contributes to phenotypic variation and could influence the spread of an introduced species in novel environments. This may occur through two different (non-mutually exclusive) mechanisms. Individuals may benefit from existing (and heritable) epigenetic diversity or de novo epigenetic marks may increase in response to the new environment; both mechanisms might increase flexibility in new environments. Although epigenetic changes in invasive plants have been described, no data yet exist on the epigenetic changes throughout a range expansion of a vertebrate. Here, we used methylation sensitive-amplified fragment length polymorphism to explore genome-wide patterns of methylation in an expanding population of house sparrows (Passer domesticus). House sparrows were introduced to Kenya in the 1950s and have significant

---

3 Portions of these results have been previously published (Liebl et al, 2013) and are utilized with permission of the publisher (Appendix B). Andrea L. Liebl, Aaron W. Schrey, Christina L. Richards, and Lynn B. Martin designed the research and contributed analytic tools. The research was performed and analyzed by Andrea L. Liebl and Aaron W. Schrey.
phenotypic variation dependent on the time since colonization. We found that Kenyan house sparrows had high levels of variation in methylation across the genome. Interestingly, there was a significant, potentially compensatory relationship between epigenetic and genetic diversity: epigenetic diversity was negatively correlated with genetic diversity and positively correlated with inbreeding across the range expansion. Thus, methylation may increase phenotypic variation and/or plasticity in response to new environments and therefore be an important source of inter-individual variation for adaptation in these environments, particularly over the short time scales over which invasions occur.

**Introduction**

Introduced species offer an opportunity to study the evolution of small populations in novel, or changing environments. The expansion of newly introduced populations, which often are small and presumably not adapted to their new habitat, is somewhat of a genetic paradox (Allendorf and Lundquist 2003). The reduction of genetic diversity in small populations can limit population growth and the ability to evolve in novel environments, as is often observed in conservation biology (Allendorf and Lundquist 2003). However, many introduced species are successful in their new environments despite few initial colonists, bottlenecks, and founder effects. Epigenetic mechanisms, such as DNA methylation, may contribute to the success of introduced species in novel habitats (Perez et al. 2006; Rando and Verstrepen 2007; Herrera et al. 2012; Richards et al. 2012; Schrey et al. 2012; Zhang et al. 2013).
Research in both plants and animals (Dolinoy et al. 2007; Kucharski et al. 2008; Bossdorf et al. 2010; Zhang et al. 2013) suggest that epigenetic processes partially mediate environmentally induced phenotypic variation (Vogt et al. 2008; Angers et al. 2010; Gao et al. 2010; Richards et al. 2010). Epigenetic marks can be induced or removed in response to environmental cues throughout the lifetime of an individual (Angers et al. 2010; Verhoeven et al. 2010). Changes in methylation not only influence mean values of traits, but also the plasticity of certain traits (Bossdorf et al. 2010). Increased plasticity and the fact that epigenetic changes can occur within the lifetime of an individual indicate that epigenetic mechanisms may mediate changes on a finer timescale than genomic evolution; this ability to finely-tune a phenotype in response to environmental cues might be especially important in novel and/or changing habitats. Given the rapid response of epigenetic changes, they may increase the fitness of local populations (Herrera and Bazaga 2011). Additionally, variation in methylation can be greater than (Richards et al. 2012; Schrey et al. 2012), and independent of genetic variation (Herrera and Bazaga 2011), indicating that changes in epigenetic diversity may occur despite reductions in genetic diversity (e.g. as might be expected following a bottleneck). There have been few MS-AFLP studies focusing on differences in methylation in introduced species (but see (Chwedorzewska and Bednarek 2012; Richards et al. 2012; Schrey et al. 2012) and few of natural variation in MS-AFLP among vertebrates (but see (Massicotte et al. 2011; Massicotte and Angers 2012; Schrey et al. 2012), but these studies indicate that DNA methylation may play an important role in the adaptation of introduced vertebrates in novel environments.
House sparrows (*Passer domesticus*) are one of the world’s most broadly distributed vertebrate species and have been introduced to much of their range (Anderson 2006). Phenotypic differences among populations indicate they are able to circumvent the loss of genetic diversity associated with an introduction (e.g. (Johnston and Selander 1973; Martin and Fitzgerald 2005; Martin et al. 2005). Epigenetically, Schrey et al. (2012) revealed greater methylation at two loci in a recently introduced population of house sparrows compared to one that had colonized earlier; this may indicate that methylation is an important facet of house sparrow invasion and range expansion.

One of the most recently introduced house sparrow populations was to Mombasa, Kenya (MO) in the 1950s (Anderson 2006); from MO, despite a small founding population (Anderson 2006), house sparrows have expanded to most major cities in Kenya and, compared to other populations, Kenyan house sparrows have reduced genetic diversity (Schrey et al. 2011). Within Kenya, microsatellite-based genetic data indicate that the main expansion of house sparrows occurred along the major highway in southern Kenya (connecting MO to Nairobi (NA) and then west towards Uganda) with genetic admixture (Schrey et al. *in review*). Despite low genetic diversity, genetic admixture among cities, and a brief existence in Kenya, house sparrows exhibit phenotypic differentiation in a pattern consistent with the length of time since colonization (Liebl and Martin 2012; Liebl and Martin 2013; Martin et al. *in review*). If phenotypic differentiation in Kenyan house sparrows was solely dependent on underlying genetic differentiation, we would expect no clear pattern of phenotypic variation to emerge; this outcome is, however, contrary to what we find.
Here, we screened natural variation in DNA methylation among cities throughout the range expansion of house sparrows in Kenya. We described epigenetic diversity and differentiation among sites in relation to the initial point of introduction, MO. Our goal was to determine whether variation and differentiation exists in genome-wide DNA methylation among individuals and/or cities across Kenya. By further extending the scope of our analysis by comparing our results with that of a microsatellite-based genetic study of the same individuals (Schrey et al. in review), we could determine whether epigenetic and genetic variation are related in Kenyan house sparrows. Thus, we were able to test the hypothesis that epigenetic diversity might act as a compensatory mechanism for reduced heterozygosity and increased inbreeding, as is often seen after an introduction.

Materials and Methods

Collection of epigenetic data

We screened epigenetic variation in 43 individuals from seven cities across Kenya (Table 4.1; Figure 2.1): Mombasa (MO), Malindi/Watamu (MA), Garsen (GA), Nairobi (NA), Nyeri (NY), Nanyuki (NN), and Kakamega (KA). Individuals were bled at capture and blood was preserved in RNAlater (Qiagen) at room temperature for up to three months before being frozen (-20°C). DNA was extracted from 50 μl of sample mixture (i.e. RNAlater plus blood) using a standard phenol:chloroform protocol for DNA extraction (Russell and Sambrook 2001).

We performed methylation sensitive-amplified fragment length polymorphism (MS-AFLP; (Reyna-Lopez et al. 1997)) with the protocol described by Schrey et al.
(2012), which modified an AFLP protocol by substituting methylation-sensitive isoschizomeric enzymes \textit{MspI} and \textit{HpaII} for \textit{MseI}. \textit{MspI} and \textit{HpaII} have different sensitivities to cytosine methylation. Thus, by performing the protocol in parallel for each enzyme for every individual, we could identify the state of methylation at each restriction site. We used one primer combination for selective PCR (Schrey et al. 2012) at a final volume of 10 µL; the thermal cycle was: 95°C for 2 m, 95°C for 30 s, 53°C for 30 s, 72°C for 30 s, and 70°C for 5 m, repeated 40 times. We used PEAKSCANNER v 1.0 (Applied Biosystems) to analyze resultant gel files and define fragment sizes. We duplicated the entire protocol for at least two individuals from each city to identify bands that consistently occurred, and eliminated bands that inconsistently amplified or occurred at highly variable intensities. We pooled data into two categories: methylated (Type II, Type III) or not methylated (Type I) (Salmon et al. 2008). Type IV epigenetic variation was not included in the analysis, as it can be generated either by an epigenetic modification or a change in DNA sequence at the restriction site.

\textit{Analysis of epigenetic data}

All analyses were conducted using a binary haplotype binding pattern (above; 1 for methylated, 0 for not methylated) for a total of 31 banding sites. Due to low sample sizes of either sex from each city, we pooled all individuals for all analyses. We calculated haplotype diversity \((h)\) and the proportion of polymorphic loci (%\(P\)) with GENALEX-6 (Peakall and Smouse 2006) to characterize epigenetic diversity. These estimates were compared with distance from MO (DFM), the site of initial introduction, as a proxy of time since colonization (Table 1) (Liebl and Martin 2012). We also
calculated $\Phi_{ST}$ among sites using the AMOVA framework of GENALEX-6 to estimate the amount of epigenetic differentiation among cities. $\Phi_{ST}$ was calculated over all loci and locus-by-locus; statistical significance was estimated after 9999 permutations. We used a sequential Bonferroni correction of $\alpha = 0.05$ for multiple tests (Rice 1989).

**Contrasting epigenetic and genetic characteristics**

As the genetic and epigenetic estimates of diversity and differentiation are fundamentally different, we could not directly compare them; therefore, we compared the pattern of change in these estimates throughout the range expansion. To determine whether epigenetic and genetic variation and differentiation were similar, we compared the MS-AFLP-based diversity ($h$ and $\%P$) and differentiation ($\Phi_{ST}$) to genetic characteristics of microsatellite loci of the same individuals, described elsewhere (Table 4.1) (Schrey et al. *in review*). Specifically, we compared the MS-AFLP results to observed heterozygosity ($H_O$; which increased with DFM), expected heterozygosity ($H_E$; which tended to decrease with DFM), inbreeding ($F_{IS}$; which decreased with DFM), and $F_{ST}$ (which detected significant genetic differentiation among all seven cities) (Schrey et al. *in review*).

To address the possibility of type II error in the $\Phi_{ST}$ estimates of differentiation at MS-AFLP loci, we performed a power analysis following Cohen’s (1988) proportion of variance method for $F$ statistics. We determined the power of our MS-AFLP data to detect differentiation among Kenyan house sparrows from the effect-size corresponding to the amount of genetic differentiation detected with microsatellites ($\Phi_{ST} = 0.127$). We
then estimated power from the effect-size, with degrees of freedom = 6, \( n = 42 \) and 44 (bracketing the actual \( n = 43 \)), and \( \alpha = 0.05 \).

**Results**

*Observed epigenetic diversity and differentiation*

There was a great deal of variation in DNA methylation among individuals, such that all individuals had unique epigenotypes. Among cities, \( h \) ranged from 0.28 to 0.44 and \%\( P \) ranged from 0.58 to 0.90 (Table 4.1). There was no relationship between \( h \) and DFM, \%\( P \) and DFM, nor was there epigenetic differentiation among any of the cities; over all loci \( \Phi_{ST} = 0.004, P = 0.41 \), and locus-by-locus \( \Phi_{ST} \) ranged from -0.14 to 0.19. No locus-by-locus estimate of \( \Phi_{ST} \) was significant. The power analysis indicated that the MS-AFLP data had power >0.995 to detect a similar level of differentiation as that detected among cites with microsatellites.

*Contrasting epigenetic and genetic characteristics*

We detected a significant relationship between epigenetic and genetic diversity (Figure 4.1). Epigenetic diversity (both \( h \) and \%\( P \)) was negatively correlated with \( H_O \) \( (r = -0.83, P = 0.01; \) and \( r = -0.82, P = 0.01, \) respectively); further, epigenetic diversity was positively correlated with \( F_{IS} \) \( (h \) and \( F_{IS}: r = 0.86, P = 0.007; \) and \%\( P \) and \( F_{IS}: r = 0.89, P = 0.004 \). There was only a marginal relationship between epigenetic diversity and \( H_E \) \( (h \) and \( H_E: r = 0.64, P = 0.06; \) \%\( P \) and \( H_E: r = 0.50, P = 0.13 \).
Discussion

Epigenetic mechanisms likely impact the evolutionary potential of wild populations (Jablonka and Raz 2009; Bossdorf et al. 2010); however, this possibility has rarely been tested in wild vertebrates (but see (Massicotte et al. 2011; Morán and Pérez-Figueroa 2011; Liu et al. 2012; Schrey et al. 2012). Epigenetic variation could be particularly important in introduced populations, which must adjust to novel habitats with relatively low levels of genetic variation (Richards et al. 2012). We have already shown that Kenyan house sparrows have lower levels of genetic diversity than native and longer-established populations (Schrey et al. 2011) and also that they have higher levels of methylation at some loci compared to another, longer established population (Schrey et al. 2012). Although we did not find a pattern of epigenetic differentiation throughout the range in Kenya, we did find a great deal of variation in methylation among individuals. Additionally, we detected a significant, negative relationship between epigenetic and genetic diversity. Our results suggest that following introduction to a novel habitat, epigenetic diversity may increase in areas where genetic diversity is low and inbreeding occurs.

Epigenetic diversity

Considerable phenotypic diversity exists among Kenyan house sparrows. Individuals on the edge of the range are more exploratory (Liebl and Martin 2012), have a greater corticosterone response to stressors (Liebl and Martin 2012; Liebl and Martin 2013), and regulate immune responses differently (Martin et al. in review) than do birds from the oldest Kenyan sites. However, reduced genetic diversity and overall genetic
admixture throughout the expanded range suggest that selection on genetic polymorphisms alone cannot explain these patterns. Although we did not detect epigenetic differentiation among cities throughout the expanded range (contrary to genetic differentiation) (Schrey et al. in review), power analyses indicate that if such variation existed (at least to the same degree as with microsatellites), we would have detected it. The lack of differentiation and overall high variability among individuals suggest that individuals maintain high levels of epigenetic variability or preserve the ability to change epigenetic marks in response to the environment.

Interestingly, we detected a significant, potentially compensatory, relationship between epigenetic diversity and genetic diversity: epigenetic diversity increased as observed heterozygosity decreased and the inbreeding coefficient increased. Epigenetic variation likely contributes to existing genomic variation. Further, when genomic variation is low, epigenetic marks may be an especially important source of phenotypic variation (Geoghegan and Spencer 2012). In fact, epimutations have been implicated as a faster source of adaptation than genetic mutations (Jablonka and Lamb 1989; Jablonka and Lamb 1998); organisms may use the additional variation afforded by epigenetic mechanisms as a bet-hedging strategy in unknown environments (Jablonka and Lamb 1989; Jablonka and Lamb 1998; Pál and Miklós 1999). We predict that when genetic diversity is low (through the loss of allelic diversity or inbreeding), greater epigenetic diversity may rescue phenotypes through increased phenotypic variation and/or plasticity in response to new environments (but see Vergeer and Ouborg 2012).

Although our design cannot discriminate the three kinds of epigenetic variation described by Richards (2006): obligatory (dependent on genetic variation), facilitated
(directed by genetic variation), and pure (generated by environmental stimuli), we predict all three may play a role in range expansions. Pure epigenetic variation may be particularly important when induced in direct response to the environment, which would be adaptive when environments are unpredictable and/or changing rapidly. Regardless of the type, epigenetic effects can be generated de novo in response to environmental cues that occur within the lifetime of an individual (Herrera et al. 2012) or be stably inherited from a parent (Richards 2006). Although inherited epigenetic marks may play a role in expansions of geographic ranges, particularly if those marks were determined during a generation following the initial expansion, the marks generated de novo might be more impactful as they are likely tailored to each individual’s unique developmental and environmental experience. In this respect, methylation serves as a mechanism of phenotypic plasticity (Richards et al. 2010; Herrera et al. 2012). If adaptive, these environmentally dependent effects may eventually lead to the fixation of certain traits (i.e. through genetic assimilation), but if the trait’s adaptive value is contingent on environmental context, then selection should maintain methylation that is responsive to environmental stimuli (West Eberhard 2003). Therefore, if changes in the environment occur too rapidly, or too often for inherited traits to produce adaptive outcomes, epigenetic changes may facilitate the persistence of populations (Price et al. 2003; Bonduriansky and Day 2009). Even labile epigenetic marks can alter evolution (Day and Bonduriansky 2011). In the absence of environmentally induced epigenetic variation, allele frequency could increase and eventually spread to fixation; in a constant environment, an allele influenced by environmentally induced methylation could also increase in frequency, but, if the environment changed, only the allele influenced by
environmentally induced methylation would have the ability to change, releasing further variation of that allele (Day and Bonduriansky 2011). In rapidly changing environments, selection should favor mechanisms that allow a wide variety of traits or lability in traits.

**Conclusions**

Both environmental and genomic stress stimulate epigenetic “repatterning,” which increases phenotypic variation and could lead to novel phenotypes subject to natural selection (Rapp and Wendel 2005). Individuals presumably undergo both environmental stressors (e.g. novel environment with novel resources, predators, parasites, etc.) and genomic stressors (e.g. bottlenecks, founder effects, inbreeding) during introduction into a new area and subsequent range expansion. Epigenetic mechanisms increase variation and affect adaptation and divergence of stable variants without underlying genetic variation (Kalisz and Purugganan 2004; Rapp and Wendel 2005; Jablonka and Raz 2009), and are therefore likely an important mechanism in the success of many introduced species.

**Funding**

This work was supported by the National Science Foundation (IOS-0920475 to LBM and BS-1209747 to LBM and ALL) and the University of South Florida (to CLR).
Table 4.1. Kenyan cities where house sparrows were collected and screened for variation in DNA methylation (with abbreviations, Abb.). The distance (km) from Mombasa (DFM), number of individuals screened (N), individual information (i.e. number of males, females, and immature individuals (M/F/I)), epigenetic diversity (as haplotype diversity, $h$ and percentage of polymorphic loci, $\%P$; determined using GENALEX-6), along with a summary of data from microsatellite-based genetic data (observed heterozygosity, $H_O$, expected heterozygosity, $H_E$, and the inbreeding coefficient, $F_{IS}$; Schrey et al., in review) are provided for each city.

<table>
<thead>
<tr>
<th>City</th>
<th>Abb.</th>
<th>DFM</th>
<th>N</th>
<th>M/F/I</th>
<th>$h$</th>
<th>$%P$</th>
<th>$H_O$</th>
<th>$H_E$</th>
<th>$F_{IS}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mombasa</td>
<td>MO</td>
<td>0</td>
<td>5</td>
<td>1/2/2</td>
<td>0.28</td>
<td>58.06</td>
<td>0.82</td>
<td>0.75</td>
<td>-0.24</td>
</tr>
<tr>
<td>Malindi</td>
<td>MA</td>
<td>120</td>
<td>6</td>
<td>4/2/0</td>
<td>0.37</td>
<td>83.87</td>
<td>0.69</td>
<td>0.82</td>
<td>0.06</td>
</tr>
<tr>
<td>Garsen</td>
<td>GA</td>
<td>230</td>
<td>4</td>
<td>3/1/0</td>
<td>0.44</td>
<td>80.65</td>
<td>0.64</td>
<td>0.82</td>
<td>0.10</td>
</tr>
<tr>
<td>Nairobi</td>
<td>NA</td>
<td>500</td>
<td>8</td>
<td>3/2/3</td>
<td>0.34</td>
<td>77.42</td>
<td>0.68</td>
<td>0.71</td>
<td>-0.03</td>
</tr>
<tr>
<td>Nyeri</td>
<td>NY</td>
<td>630</td>
<td>5</td>
<td>2/3/0</td>
<td>0.32</td>
<td>61.29</td>
<td>0.80</td>
<td>0.71</td>
<td>-0.26</td>
</tr>
<tr>
<td>Nanyuki</td>
<td>NN</td>
<td>675</td>
<td>9</td>
<td>4/5/0</td>
<td>0.41</td>
<td>90.32</td>
<td>0.71</td>
<td>0.75</td>
<td>-0.01</td>
</tr>
<tr>
<td>Kakamega</td>
<td>KA</td>
<td>885</td>
<td>6</td>
<td>4/2/0</td>
<td>0.39</td>
<td>80.65</td>
<td>0.69</td>
<td>0.80</td>
<td>0.01</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>43</td>
<td>21/17/5</td>
<td>0.36</td>
<td>76.04</td>
<td>0.72</td>
<td>0.77</td>
<td>-0.05</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1. Scatterplots of genetic diversity (H\textsubscript{O} and F\textsubscript{IS}) versus epigenetic diversity (h).

Epigenetic diversity is related to genetic diversity among house sparrows from seven Kenyan cities screened at both MS-AFLP and microsatellite loci. (a) Epigenetic diversity was negatively correlated with observed heterozygosity (H\textsubscript{O}): as H\textsubscript{O} increased, both haplotype diversity (h) and the proportion of polymorphic loci (%P) decreased ($r = -0.83$, $P = 0.01$ and $r = -0.82$, $P = 0.01$ respectively). (b) Epigenetic diversity was positively correlated with the inbreeding coefficient (F\textsubscript{IS}): as F\textsubscript{IS} increased, both h and %P increased ($r = 0.86$, $P = 0.007$ and $r = 0.89$, $P = 0.004$, respectively). Only h is shown here due to the high correlations between h and %P.
CHAPTER FIVE: CONCLUSIONS

The studies I described in this dissertation examined physiological and behavioral changes as they occur throughout a range expansion of an introduced vertebrate, the house sparrow (*Passer domesticus*). The changes reported here may be adaptive in response to variable environments at different points of the range and may be indicative of what changes might be expected in other species expanding their ranges following an introduction or in response to environmental change (e.g. climate change, urbanization).

Physiologically, I reported that individual house sparrows at a range edge release significantly more glucocorticoids (GCs) in response to restraint than those from older, longer established populations and had a lower mineralocorticoid receptor (MR) density relative to glucocorticoid receptors (GR) in the hippocampus. Behaviorally, I reported that house sparrows captured from the edge of their range were significantly more exploratory than those from Mombasa. Finally, as the genetic diversity of Kenyan house sparrows is low compared to other house sparrow populations (Schrey et al. 2011), I also compared epigenetic to genetic diversity in Kenyan house sparrows and found that epigenetic variability increased as genetic diversity decreased and inbreeding increased. Although a direct comparison between individual phenotype and epigenetic pattern was not possible here (not all individuals used to describe physiological and behavioral patterns were used in the epigenetic study), it would be interesting in the future to
determine if the phenotypes described here are related to specific epigenotypes. Below, I discuss potential avenues of research following the results I reported here.

Few species have been studied in the context of range expansion, but based on the patterns documented here and elsewhere (e.g. (Duckworth and Badyaev 2007; Gunnarsson et al. 2012), I predict phenotypic differences among individuals throughout a range exist in many species. It is even possible that the traits described here are characteristic of range expansions generally; more work, however, needs to be done to determine the broad applicability of these results to other organisms. If traits common to many range expansions could be identified, however, these traits could be used as markers of invasion potential to determine which species, populations, or individuals are most likely to be successful and/or spread following an introduction; in other words, these traits could be used to identify high risk species, populations, or individuals so that management efforts could be targeted most appropriately to limit ecosystem (and economic) destruction.

Future work should identify the fitness benefits of each phenotype in different environments (i.e. range edge or range center) and determine whether an adaptive value of certain phenotypes exists and whether that value is dependent on the age of the population. If high GC responsiveness, low MR:GR ratio, high exploration, and high epigenetic variability are indeed adaptive in novel environments, it may prove to be beneficial to use these traits in conservation efforts, particularly in organisms facing anthropogenic shifts and changes to the environment. It may be possible to manipulate traits (e.g. using receptor antagonists or methylation inhibitors (e.g. Trichostatin A)), promoting individual adaptability in novel, changing, and unpredictable environments.
The patterns described in this dissertation are interesting, but one question left unanswered is how these patterns emerge; determining the mechanism(s) through which such rapid changes are made throughout the range expansion could be insightful in invasion biology, conservation biology, and studies of adaptation generally. Rapid changes such as those described here could be a result of rapid genetic evolution, developmental effects (e.g. maternally-derived hormones) (Mousseau and Fox 1998), and/or individual flexibility in response to environmental cues (Cheviron et al. 2008). Given reduced genetic diversity compared to other house sparrow populations and overall admixture throughout the range expansion, rapid genetic evolution is an unlikely (albeit not impossible) explanation for the physiological and behavioral differences observed among Kenyan house sparrows. Other more likely (but not mutually exclusive) explanations include developmental effects and phenotypic flexibility.

The presumed mode of expansion in Kenyan house sparrows is via human-mediated mechanisms (i.e. accident movement with commercial goods by truck or train; (Schrey et al. in review)), thus any individual could be moved to a novel area, just by foraging on a particular truck (i.e. individuals foraging inside the trailer of a truck at the time of loading could be shut in and transported with the goods). As such, it is possible that individuals with a higher propensity to forage on or near trucks are those more likely to be more exploratory and have a higher, more efficient GC response, and therefore be most suited for life at the range edge. This would be predicted if evolution maintained separate dispersers and philopatric individuals at the population level as has been seen elsewhere (Duckworth and Badyaev 2007) and it might be interesting to test differences in behavior and physiology between birds caught on and near trucks compared to those
caught away from loading areas. However, another explanation is that Kenyan house sparrows maintain the flexibility necessary to adjust their phenotype rapidly to cues in novel environments. Individuals with the ability to rapidly adjust to different environments are more likely to survive in novel or unpredictable environments. Further, although flexibility might allow individuals who disperse to new areas to best match their new environment, canalization of those phenotypes (Waddington 1942; West Eberhard 2003) would ensure the persistence of those phenotypes at the range edge.

Developmental effects likely contribute to the canalization of those phenotypes and maternal influences during development have been shown previously to be influential in both range expansions (Duckworth and Badyaev 2007; Duckworth 2008; Duckworth 2009) as well as GC responsiveness (Meaney 2001; Kitaysky et al. 2003; Cyr and Romero 2007; Love and Williams 2008). Interestingly, in Kenyan house sparrows, differences in GC response only occurred during the breeding season (not when birds were molting)- when mothers would be most influential on offspring (through hormone deposition in yolks or resource allocation to nestlings).

Based on the patterns documented here and elsewhere (e.g. (Duckworth and Badyaev 2007; Gunnarsson et al. 2012), individuals at a range edge are phenotypically different than those from the center of the range. Although the traits described here may be characteristic of range expansions generally and therefore could be used as an “invasive marker”, more work needs to be done to determine the applicability of these results in other organisms. However, I believe to best understand how to limit the range expansions of other introduced species as well as promote the survival of threatened
species in response to global climate change, we need to better understand how phenotypic changes are made in response to variable environments.
REFERENCES CITED


Bossdorf, O., D. Arcuri, C. L. Richards and M. Pigliucci. 2010. Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in arabidopsis thaliana.


Martin, L. B., C. A. C. Coon, A. L. Liebl and A. W. Schrey. in review. Surveillance for microbes and range expansion in house sparrows


67


APPENDICES

Appendix A: Permission of use, Royal Society Publishing

Relevant sections are in bold, underlined text.

Licence to Publish

Notice: You are the person first named under the heading ‘My Authors’ at stage 3 of the online process to ‘Submit a Manuscript’ (referred to as “You/Your”). We are the Royal Society, a body incorporated by royal charter, with its place of business at 6-9 Carlton House Terrace, London SW1Y 5AG (referred to as “We/Us/Our”).

You have indicated Your intention to upload the article more fully detailed at stage 1 of the online submission process (the “Article”) to be considered for publication by Us. In order to publish Your article We need You to grant Us a licence to publish. This licence also sets out Your rights regarding use of Preprints, Author Generated Postprints and the Definitive Published Version of the Article (as defined below). Please read the terms of this licence carefully before uploading the Article. Clicking on the button marked ‘Yes’ next to the question “Do you accept the terms and conditions of the Royal Society’s Licence to Publish?” at stage 5 of the online submission process will be taken as assurance that You have read, agree to grant and have the right to grant this licence.

Definitions

“Preprints” - the un-refereed version of the Article;

“Author Generated Postprint” - Your personal copy of the revised version of the Article as accepted by Us;

“Definitive Published Version” - the citable version of the Article produced by Us after peer review, copy editing and print and electronic production.
1. By clicking the button marked ‘Yes’ next to the question “Do you accept the terms and conditions of the Royal Society’s Licence to Publish?” at stage 5 of the online submission process, and uploading the Article, You grant to Us for the full term of copyright in the Article and any extensions thereto the exclusive right throughout the world to edit, adapt, translate, publish reproduce, distribute and display the Article in printed, electronic or any other medium and format whether now known or yet to be developed; and

2. You warrant to Us that:
a) the Article is Your original work, has not previously been published and is not currently under consideration for publication by any other entity;

b) in the case of a multi-authored Article, You have obtained written authorisation from all the co-authors of the Article (if any) to grant this licence to Us on their behalf as their agent, and You will supply a copy of the same to Us if We so request;

c) where any copyright material has been included in the Article which has been sourced from third parties (eg illustrations, photographs, charts or maps), You have obtained all necessary written authorisations for the reproduction and distribution of these materials as part of the Article throughout the world, in all languages and in all media and formats whether now known or yet to be developed and You will supply a copy of the same to Us if We so request;

d) if copyright in the Article is owned by any third party, whether Your employer or someone to whom You have assigned Your rights, You have obtained written authorisation from such copyright owner to grant this licence to Us on their behalf as their agent and will supply a copy of the same to Us if We so request; and

e) the Article does not contain anything which is obscene, defamatory, libellous, infringes any right of privacy or any intellectual property right (including without limitation rights in patents, copyright or trade marks) or any other rights of any person or entity, or is otherwise unlawful.

3. You assert Your moral right to be identified as the Author or co-author of the Article (as applicable). If Your Article is published, We will provide You with a PDF copy of the published Article.

4. If You decide to make the Definitive Published Version of the Article open access, this will be under a Creative Commons BY licence*,

You shall pay to Us the relevant fee and We shall make the Article so available from the later of the date of receipt of the relevant fee or the date of first publication of the Article.
You warrant to Us:

in the case of a multi-authored Article, You have obtained written authorisation from all the co-authors of the Article (if any) to grant a CC-BY licence to Us on their behalf as their agent, and You will supply a copy of the same to Us if We so request;

where any copyright material has been included in the Article which has been sourced from third parties (eg illustrations, photographs, charts or maps), You have;
either obtained all necessary written authorisations for the reproduction and distribution of these materials to grant a CC-BY licence to Us and You will supply a copy of the same to Us if We so request; or clearly indicate that material which is not under a CC-BY licence.

if copyright in the Article is owned by any third party, whether Your employer or someone to whom You have assigned Your rights, You have obtained written authorisation from such copyright owner to grant a CC-BY licence to Us on their behalf as their agent and will supply a copy of the same to Us if We so request.

5. **You retain copyright in the Article.** However, You authorise Us to act on Your behalf to defend Your copyright in the Article should anyone infringe it, and to retain half of any damages awarded after deducting Our costs.

6. **You retain the right to use the Article in the following ways, provided that you acknowledge the Definitive Published Version of the article by placing the full bibliographic reference and URL of the relevant journal homepage close to the title of the Article:**

a) In relation to the Preprint, Author Generated Postprint and Definitive Published Version of the Article, You are free to: make copies for Your own personal use; use the Article for the internal teaching purposes of Your own institution or company; and make and distribute copies (including through e-mail) of the Article to research colleagues, for personal use by such colleagues on a non-commercial, non-systematic basis.

b) In relation to the Preprint version only, You are free to post it on web sites, including electronic preprint servers.

**When the Definitive Published Version of the article is published the Author must acknowledges it by placing the full bibliographic reference and URL of the relevant journal homepage close to the title of the Article.**

c) **In relation to the Author Generated Postprint only, You are free to:** post it on Your personal or institutional web site and load it onto an institutional or not for profit repository no earlier than 12 months from the date of first publication of the Definitive Published Version provided that a link to the Definitive Published Version is included;
use it in printed compilations of Your work subsequent to publication of the Definitive Published Version of the Article, expand the Article into book-length form, and/or otherwise re-use portions of the Author Generated Postprint of the Article in other works. You are also free to present the Article at a meeting or conference and to disseminate copies of such Article to the delegates attending such meeting or conference and/or to use the Author Generated Postprint in a thesis or dissertation (provided that this is not to be published commercially).

7. You agree to indemnify Us and keep Us indemnified against all losses, costs and expenses (including legal costs and expenses) arising from any claims made against Us by third parties concerning the authorship of the Article, the right to publish the Article or the infringement of any third party’s rights.

8. We are entitled to assign Our rights under this licence to any third party without giving notice to You.

9. No change or modification of this licence will be valid unless confirmed in writing by Us.

10. Failure or delay by Us to exercise any right or remedy under this Agreement shall not be deemed to be a waiver of that right or remedy, or prevent Us from exercising that or any other right or remedy on any occasion.

11. This licence is governed by English law and the parties hereby submit to the non-exclusive jurisdiction of the English courts.

12. This license is terminated in case the article is rejected for publication or the author withdraws the article for consideration for publication before publication has occurred.
Appendix B: Permission of use, Oxford Journals

Relevant sections are in bold, underlined text.

Publication Rights Policies

For the majority of journals published by Oxford University Press, we have a policy of acquiring a sole and exclusive licence for all published content, rather than asking authors to transfer ownership of their copyright, which has been common practice in the past. We believe this policy more carefully balances the interests of our authors with our need to maintain the viability and reputation of the journals through which our authors are accorded status, recognition and widespread distribution. In developing this policy we have been guided by the following principles:

- As a university press and not-for-profit academic publisher, we rely heavily on the good relationships we have with our authors. Having a licensing policy which enables an author to be identified as the owner of the copyright in an article is one of the key ways of demonstrating how highly we value these relationships.
- An exclusive licence enables the centralised and efficient management of permissions and licencing, ensuring the widest dissemination of the content through intermediaries;
- Exclusive rights also enable OUP to take measures on behalf of our authors against infringement, inappropriate use of an article, libel or plagiarism;
- At the same time, by maintaining exclusive rights, in all media for all published content, we can monitor and uphold the integrity of an article once refereed and accepted for publication to be maintained;
Where to get a copy of the Licence to Publish

OUP cannot publish your article until a completed licence form has been received. You should receive a form as soon as your article is accepted for publication.

Footnotes to this section

1. A small number of OUP Journals still have a policy of requesting a full Assignment of Copyright. If unclear about the policy of the Journal concerned, please contact the Editorial office to clarify.

Government employees

- If you are or were a UK Crown servant and the article has been written in that capacity, we have an arrangement with HMSO to enable us to publish it while acknowledging that it is Crown Copyright. Please inform the Editorial office or Oxford University Press at the time of acceptance or as soon as possible that the article is Crown Copyright, so that we can ensure the appropriate acknowledgement and copyright line are used, as required by our arrangement with HMSO.
- If you are a US Government employee and the article has been written in that capacity, we acknowledge that the Licence to Publish applies only to the extent allowable by US law.

Re-use of third party content as part of your Oxford Journals article

- As part of your article, you may wish to reuse material sourced from third parties such as other publishers, authors, museums, art galleries etc. To assist with this process, we have a Permission Request form and accompanying Guidelines that specifies the rights required in order for third party material to be published as part of your Article. For a copy of this form, please email.
- Responsibility for clearing these third party permissions must be borne by the Author, and this process completed as soon as possible - preferably before acceptance of the manuscript, but if not possible, before the Article reaches the Production stage of the process.

Rights retained by ALL Oxford Journal Authors

- The right, after publication by Oxford Journals, to use all or part of the Article and abstract, for their own personal use, including their own classroom teaching purposes;
- The right, after publication by Oxford Journals, to use all or part of the Article and abstract, in the preparation of derivative works, extension of the article into book-length or in other works, provided that a full acknowledgement is made to the original publication in the journal;
• The right to include the article in full or in part in a thesis or dissertation, provided that this not published commercially;

For the uses specified here, please note that there is no need for you to apply for written permission from Oxford University Press in advance. Please go ahead with the use ensuring that a full acknowledgment is made to the original source of the material including the journal name, volume, issue, page numbers, year of publication, title of article and to Oxford University Press and/or the learned society.

The only exception to this is for the re-use of material for commercial purposes, as defined in the information available via the above url. Permission for this kind of re-use is required and can be obtained by using Rightslink:

With Copyright Clearance Center’s Rightslink service it’s faster and easier than ever before to secure permission from OUP titles to be republished in a coursepack, book, CD-ROM/DVD, brochure or pamphlet, journal or magazine, newsletter, newspaper, make a photocopy, or translate.

• Simply visit: www.oxfordjournals.org and locate your desired content.
• Click on (Order Permissions) within the table of contents and/ or at the bottom article’s abstract to open the following page:
• Select the way you would like to reuse the content
• Create an account or login to your existing account
• Accept the terms and conditions and permission is granted

For questions about using the Rightslink service, please contact Customer Support via phone 877/622-5543 (toll free) or 978/777-9929, or email Rightslink customer care.

Preprint use of Oxford Journals content

• For the majority of Oxford Journals, prior to acceptance for publication, authors retain the right to make a pre-print [A preprint is defined here as un-refereed author version of the article] version of the article available on your own personal website and/or that of your employer and/or in free public servers of preprints and/or articles in your subject area, provided that where possible.
  o You acknowledge that the article has been accepted for publication in [Journal Title] ©: [year] [owner as specified on the article] Published by Oxford University Press [on behalf of xxxxxx]. All rights reserved.
  o Once the article has been published, we do not require that preprint versions are removed from where they are available. However, we do ask that these are not updated or replaced with the finally published version. Once an article is published, a link could be provided to the final authoritative version on the Oxford Journals Web site. Where possible, the preprint notice should be amended to:
This is an electronic version of an article published in [include the complete citation information for the final version of the Article as published in the print edition of the Journal.]

Once an article is accepted for publication, an author may not make a pre-print available as above or replace an existing pre-print with the final published version. NB There are some Oxford Journals such as the Journal of the National Cancer Institute, which do not permit any kind of preprint use. For clarification of the preprint policy for any journal please contact the Rights and New Business Development Department.

Postprint use of Oxford Journals content:

[A postprint is defined here as being the final draft author manuscript as accepted for publication, following peer review, BUT before it has undergone the copyediting and proof correction process].

We have detailed policies on the use of postprints for all of our journals. To view these for individual journals please refer to the author self archiving policies on journal homepages. If you require further information please contact the Rights and New Business Development Department.

Other uses by authors should be authorized by Oxford Journals through the Rights and New Business Development Department.

Additional Rights retained by the Author when publishing in an Oxford Open participating journal

Please note that these rights only apply to content published in an Oxford Journal on an Open Access basis in exchange for payment of an author charge. For more details about how Oxford Open works please click here.

The right to reproduce, disseminate or display articles published under this model for educational purposes, provided that:

- the original authorship is properly and fully attributed;
- the Journal and OUP are attributed as the original place of publication with the correct citation details given;
- if an article is subsequently reproduced or disseminated not in its entirety but only in part or as a derivative work this must be clearly indicated
- the right to deposit the postprint and/or URL or PDF of the finally published version of the article into an institutional or centrally organized repository, immediately upon publication

Commercial Use of Open Access version
For all articles published under a Creative Commons Attribution Licence (CC BY 3.0) or an Open Government Licence permission is not required to make any kind of commercial use of the material.

For all articles published under a Creative Commons Non-Commercial Attribution Licence (CC BY NC 3.0) or a Non-Commercial Government Licence permission is required for all commercial reuse. In order to request permission please contact the Rights and New Business Development department: you want to use and a brief description of the intended use.

Commercial re-use guidelines for open access content

Definition of commercial use: any re-use of material from the Open Access part of an Oxford Journal for the commercial gain of the user and/or their employing institution. In particular,

- re-use by a non-author/third party/other publisher of parts of or all of an article or articles in another publication (journal or book) to be sold for commercial purposes. Permission to reproduce selected figures will generally be granted free of charge, although OUP reserves the right to levy a fee for the use of these and/or the full text of an article/articles
- the proactive supply of multiple print or electronic copies of items taken from the Journal to third parties on a systematic basis for marketing purposes. Permission for this kind of reuse should be obtained from the publisher, who retains the right to levy an appropriate fee
- re-use by an author of parts of or all of an article in other publications from commercial organizations. Permission for this kind of reuse should be obtained from the publisher. We would consider this to be commercial reuse but would not normally charge a permission fee if the author is involved.

NB: Please note that any income generated from permissions granted for this kind of use will be returned directly to the journal itself in order to help minimise the costs of making content from it available on an Open Access basis.

Permissions

- All requests to reuse the article, in whole or in part, in another publication will be handled by Oxford Journals. Unless otherwise stated, any permission fees will be retained by the Journal concerned. Where possible, any requests to reproduce substantial parts of the article (including in other Oxford University Press publications) will be subject to your approval (which is deemed to be given if we have not heard from you within 4 weeks of the permission being granted).
- If copyright of the article is held by someone other than the Author, e.g. the Author's employer, Oxford Journals requires non-exclusive permission to administer any requests from third parties. Such requests will be handled in accordance with Notes 6 above.
• The Journal is registered with the Copyright Licensing Agency (London) and the Copyright Clearance Center (Danvers, Massachusetts), and other Reproduction Rights Organizations. These are non-profit organizations which offer centralised licensing arrangements for photocopying on behalf of publishers such as Oxford University Press.

• Please forward requests to re-use all or part of your article, or to use figures contained within it, to the Rights and New Business Development Department.