

**Hackle spectral characteristics and their role in mate choice in  
European starlings (*Sturnus vulgaris*)**

by

Joel Slade

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This thesis is dedicated to my grandmothers: Lois Slaunwhite and Shirley Slade (R.I.P.). For without their moral support and encouragement, I would not be the biologist I am today.

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**Abstract**

Plumage spectral characteristics are thought to play an essential role in mate choice. Male and female birds may benefit from mating outside of their social-pair bond if they obtain genetic benefits for their offspring by choosing mates with plumage that signals individual high quality. The goal of this thesis was to test the hypothesis that male and female genetic quality is signaled through hackle spectral characteristics and used in mate choice decisions by European starlings (*Sturnus vulgaris*). Hackle brightness was positively correlated with female body condition and male provisioning effort. Also males with brighter hackles sired proportionally more male offspring than males with duller hackles. Purple hackles were positively correlated with male body condition and female realized reproductive success. This study demonstrates the importance of hackle spectral quality in European starlings and the role it plays in mate choice.

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## TABLE OF CONTENTS

ABSTRACT.....	iii
ACKNOWLEDGEMENTS.....	iv
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
INTRODUCTION.....	1
MATERIALS AND METHODS.....	16
Study Site.....	16
Adult and Nestling European Starlings.....	17
Provisioning.....	18
DNA Analysis.....	19
Digestion and Extraction.....	19
DNA Quantity and Quality.....	20
Polymerase Chain Reaction (PCR).....	21
Multiplex Reactions.....	23
Genotyping.....	24
Sex Ratio Analysis.....	24
Parentage Assignment.....	25
Hackle Color Analysis.....	25
Statistical Analyses.....	26
Hackle Spectral Data.....	26
Adult Condition.....	27
Provisioning Effort.....	28
Reproductive Success.....	28
Sex Allocation.....	28
Statistical Testing.....	29
RESULTS.....	36

Hackle Spectrophotometry.....	36
Body Condition.....	37
Provisioning Effort.....	38
Reproductive Success.....	38
Sex Allocation.....	39
DISCUSSION.....	52
Hackle Spectral Characteristics.....	52
Body Condition.....	53
Provisioning Effort.....	55
Reproductive Success.....	58
Sex Allocation.....	61
Conclusions and Future Direction.....	64
Literature Cited.....	66

## **LIST OF TABLES**

<b>Table 1:</b> Characteristics of nine polymorphic loci of European starlings used in 2011 as described in Loyau et al. (2005), Rubenstein (2005), and Celis et al. (2007).....	30
<b>Table 2:</b> Characteristics of six polymorphic loci of European starlings used by Wiebe (2010) as described in Loyau et al. (2005), Rubenstein (2005), and Celis et al. (2007).....	31
<b>Table 3:</b> Characteristics of eight polymorphic loci of European starlings used by Reeve (2009) as described in Loyau et al. (2005), Rubenstein (2005), and Celis et al. (2007).....	32
<b>Table 4:</b> Characteristics of seven polymorphic loci of European starlings used in by Worth-Kerr (2008) and Wright (2008) as described in Loyau et al. (2005) and Rubenstein (2005).....	33
<b>Table 5:</b> Eigenvalues and percent of variation explained for PC1-PC21 from male European starling hackle spectral data.....	34
<b>Table 6:</b> Eigenvalues and percent of variation explained for PC1-PC21 from female European starling hackle spectral data.....	35

## LIST OF FIGURES

- Figure 1:** Eigenvectors of the first three PCs (brightness, color, and PC3) from male European starling hackle reflectance spectral data. The solid line denotes the brightness component (PC1), the dotted line denotes the color component (PC2) and the dashed line denotes PC3. The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.....40
- Figure 2:** Eigenvectors of the first two PCs (brightness and color) from female European starling hackle spectral data. The solid line denotes the brightness component (PC1) and the dotted line denotes the color component (PC2). The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.....41
- Figure 3:** Example of male hackle spectra data from six separate European starling males. The solid line denotes three sampled males with the highest brightness (PC1 scores) and the dotted line denotes three sampled males with the lowest brightness. The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.....42
- Figure 4:** Example of male hackle spectra data from six separate European starling males. The solid line denotes three males with unsaturated green hackles (high PC2 scores) that peaked in the UV and green-orange wavelengths. The dotted line denotes three males with saturated purple hackles (low PC2 scores) that peaked in the UV-violet, indigo-blue, and red wavelengths. The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.....43
- Figure 5:** Example of six male hackle spectra from six separate European starling males. The solid line denotes three males with the highest PC3 scores and dotted line denotes three males with the lowest PC3 scores. The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.....44

**Figure 6:** Example of female hackle spectra data from six separate European starling females. The solid line denotes three sampled females with the highest brightness (PC1 scores) and the dotted line denotes three sampled females with the lowest brightness. The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.....45

**Figure 7:** Example of female hackle spectra data from six separate European starling females. The solid line denotes three females with unsaturated green hackles (high PC2 scores) that peaked in the UV and green-orange wavelengths. The dotted line denotes three females with saturated purple hackles (low PC2 scores) that peaked in the UV-violet, indigo-blue, and red wavelengths. The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.....46

**Figure 8:** Hackle color (PC2 scores) of male European starlings plotted against male body condition (residuals of mass against tarsus length).....47

**Figure 9:** Hackle brightness (PC1 scores) of female European starlings plotted against female body condition (residuals of mass against tarsus length).....48

**Figure 10:** Hackle brightness (PC1 scores) of male European starlings plotted against the mean total provisionings per nestling. ....49

**Figure 11:** Hackle color (PC2 scores) of female European starlings plotted against female realized reproductive success..... 50

**Figure 12:** Hackle brightness (PC1 scores) of male European starlings plotted against the proportion of male offspring an adult male genetically produced during the breeding seasons (2007 and 2011 combined).....51

## INTRODUCTION

The phenotypic quality of a bird can be evaluated by their plumage characteristics which have evolved by mate choice in many species (Darwin 1871). This mate evaluation is important because individuals who choose a high-quality mate may gain direct and/or indirect benefits. The direct benefits of choosing a high-quality mate can include extra food provisioning, higher territory quality, enhanced vigilance, and/or protection against predators. Indirect benefits of choosing a high-quality mate would include offspring inheriting beneficial genes that increase fecundity and viability. Plumage (e.g. Hill and McGraw 2006) and courtship displays (e.g. Edler and Friedl 2012) are examples of phenotypic characteristics that birds use to assess mate quality.

There are three main hypotheses to explain avian mate choice: (1) the “good genes” hypothesis (e.g. Kirkpatrick 1996), (2) the “genetic compatibility” hypothesis (e.g. Brown 1997; Neff and Pitcher 2005), and (3) the “sexy son” hypothesis (e.g. Weatherhead and Robertson 1979). All three hypotheses focus on the genetic benefits gained by either pairing with, or securing extra-pair fertilizations from a high-quality mate.

The “good genes” hypothesis describes two mechanisms that are assumed to be driving the evolution of mate preferences in animals: the direct selection of genes associated with mate preference, and the “good genes” system (Kirkpatrick 1996). Direct selection of a preference can arise when the

preference-gene has a direct effect on viability and fecundity. The “good genes” system functions when an individual chooses a mate based on their phenotypic traits that arise from their genes, which increase total fitness. The consequential preference causes exaggeration in the trait expressed by these good genes (Kirkpatrick 1996). The exaggerations of avian secondary sexual characteristics, such as plumage, are hypothesized to have evolved through directional selection on good genes. Therefore, plumage characteristics can act as honest signals of genetic quality to potential mates (Kirkpatrick 1996).

Extra-pair copulations are assumed to be driven by female mate choice in socially monogamous passerines (e.g. Lifjeld and Robertson 1992) and can result in increased male reproductive success. The good genes hypothesis predicts that females who are paired with high-quality males are seeking fewer extra-pair copulations than females paired with low-quality males. Females that are paired with males of low genetic quality are thought to be securing extra-pair fertilizations as a manner to gain good genes for their offspring, which they would not procure if they mated solely with their low-quality social mate (e.g. Kirkpatrick 1996).

The Hamilton and Zuk (1982) hypothesis, also called parasite-mediated sexual selection, states that female birds have a preference for more elaborate males because brightness, size, and color of their plumage are condition-dependent and honest signals of genetic quality. Healthy males are able to develop the brightest plumage and produce the most energetically demanding

displays (Hamilton and Zuk 1982). For example, bright carotenoid-based plumage in male greenfinches (*Carduelis chloris*) (Saks et al. 2003), great tits (*Parus major*) (Dufva and Allander 1995; Horak et al. 2001), and Cirl buntings (*Emberiza cirrus*) (Figuerola et al. 1999) was shown to be negatively correlated with haemoparasite load. Likewise, structural-plumage brightness was negatively correlated with haemoparasite load in male satin bowerbirds (*Ptilonorhynchus violaceus*) (Doucet and Montgomerie 2003). Males of this species build and decorate bowers to attract mates, and it was shown that ultraviolet-brighter males had higher bower quality (measured by the symmetry of the bower, as well as stick size and density) and a greater number of bower decorations in comparison to duller males (Doucet and Montgomerie 2003). Male satin bowerbirds with highly decorated bowers secured more fertilizations than males with poorly decorated bowers. Therefore, it was hypothesized that plumage spectral characteristics indirectly signals an individual's immunocompetence and directly signals territory quality and arises from the expression of good genes that potential offspring could inherit.

The "genetic compatibility" hypothesis proposes that mate choice is driven by the benefit of producing genetically diverse offspring (Brown 1997). The hypothesis was invoked to explain why birds mate outside of their social-pair bond, but it does not appear to explain why secondary sexual characteristics have evolved as honest signals of mate quality. According to this hypothesis, individuals preferentially mate with members of the opposite sex that have alleles

which complement their own, ultimately resulting in their offspring becoming genetically diverse (more heterozygotic at certain loci) (Brown 1997). Compatible genes are formed when a compatible allele from one homologue is paired with a compatible allele in another homologue (e.g. gamete fusion) (Neff and Pitcher 2005). Heterozygosity at essential loci is thought to increase fitness (Brown 1997). In birds, the heterozygosity of genes plays a role in preventing inbreeding depression (e.g. Varian-Ramos and Webster 2012) and enhancing offspring immunity (e.g. major histocompatibility complex (MHC) genes (Brown 1997; Neff and Pitcher 2005; Cutrera et al. 2012)). Heterozygotic offspring are thought to be fitter than homozygotic offspring (e.g. Amos et al. 2001; Coltman and Slate 2003; Foerster et al. 2003; Mulard et al. 2009; Varian-Ramos and Webster 2012).

Birds may be of high genetic quality and provide direct benefits such as food resources, good territory, and provide excellent care to their mate and offspring, but may not be genetically compatible. Extra-pair copulations (EPCs) are hypothesized to have evolved to reduce inbreeding depression by increasing the heterozygosity of offspring if a female is paired with a genetically incompatible social-male. Blue tits (*Cyanistes caeruleus*) (Foerster et al. 2003) and red-backed fairy wrens (*Malurus melanocephalus*) (Varian-Ramos and Webster 2012) have been shown to acquire EPCs to decrease the chance of inbreeding. Therefore, the “genetic compatibility” hypothesis provides an alternative explanation to the “good genes” hypothesis as to why birds may mate outside of their social-pair bond.

The “sexy son” (Weatherhead and Robertson 1979) or the Fisherian runaway hypothesis proposes that in polygynous species, females mate with attractive males to increase the chance of their offspring inheriting mate-attracting genes (Fisher 1930). This hypothesis predicts that a female’s preference for a male is solely based on his attractiveness and not on his viability (Searcy and Yasukawa 1981) and that this attractiveness is a heritable trait passed on to the offspring (or in the case of female offspring, the preference for this trait is passed on to them) (e.g. Weatherhead and Robertson 1979; Alatalo and Lundberg 1986; Gwinner and Schwabl 2005). Weatherhead and Robertson (1979) found female red-winged blackbirds (*Agelaius phoeniceus*) preferentially mated with males based on their courtship intensity and not their territory quality (Weatherhead and Robertson 1977). A later study suggested males with the highest courtship intensity had the highest female nesting density, but these females had surprisingly low reproductive success in comparison to females nesting in low densities (Searcy and Yasukawa 1981). The negative correlation between nesting density and reproductive success was inferred to mean that females were not increasing their own reproductive success when choosing “sexy” males (those with high courtship intensity), but instead resulted in a trade-off between choosing attractive mates that did not provide an ample territory and increasing their evolutionary fitness. If a female mated with an attractive male then it is predicted that male offspring would inherit the mate-attracting traits from their father and female offspring would inherit the preference for these traits from their

mother. Consequential attractive sons would have an advantage over unattractive males in the population as they would acquire a greater number of extra-pair copulations because of their desirability as mates (Dawkins 1976).

A recent study provided support for the “sexy son” hypothesis in terms of finding that females preferred to mate with more territorial males. Sons of polygynous male European starlings (*Sturnus vulgaris*) inherited territoriality and aggression from their father, which helped them to acquire more nest sites and to better attract females than did sons of socially monogamous fathers (Gwinner and Schwabl 2005). However, studies have not been able to provide support that male plumage has evolved based on the predictions of the “sexy son” hypothesis. For example, secondary pied flycatcher (*Ficedula hypoleuca*) females that mated with highly ornamented males had a lower reproductive success than primary females because the males only fed primary nestlings (Alatalo and Lundberg 1986). The authors, however, did not examine realized reproductive success (genetically related offspring found in their nest and in the nests of others) in their study. The female pied flycatchers paired with unattractive males would still have an increase in evolutionary fitness by having a male help care for her young. Thus, it is more important to have an unattractive primary mate that provides care to nestlings than to have an attractive secondary mate that does not. Overall, there is little support for the “sexy son” hypothesis to explain the evolution of exaggerated secondary sexual characteristics used in mate choice.

The avian-visual spectrum (300-700nm) fundamentally drives the evolution of structural plumage spectral characteristics used in mate choice (Bennett et al. 1996; Bennett et al. 1997; Johnsen et al. 1998; Greenwood et al. 2002; Maddocks et al. 2002; Doucet and Montgomerie 2003; Siefferman and Hill 2005b; Evans et al. 2006; Nolan et al. 2010). Humans can only discriminate colors by neuronally comparing signals from three main photoreceptors in their retina: blue (440-490nm), green (520-570nm), and yellow-red (570-740nm) (Hill and McGraw 2006). However, birds can discriminate colors by neuronally comparing signals from four main photoreceptors in their retina: the three above and ultraviolet (UV) (300-400nm) (Hart et al. 1998; Hill and McGraw 2006). The physical interactions within feathers reflect light in the human-visual spectrum but can also produce colors in the UV-spectrum. Recent technology has allowed scientists to glimpse at how birds view short- and long- wavelength coloration (300-700nm) (Hart et al. 1998). Short-wavelength (UV-blue) coloration is more prevalent in birds than in any other vertebrate (Doucet and Meadows 2009), and a variety of birds display iridescent and non-iridescent signaling in the short-wavelength range. Feather barb and barbule structure are the main contributors to iridescent and non-iridescent signaling in birds with structurally based color (Fitzpatrick 1998; Shawkey et al. 2005; Eliason and Shawkey 2011). Physical interactions at the boundaries of feathers cause light interference and result in different refractive indices. Certain wavelengths interfere to generate colors depending on the feather microstructure, while other wavelengths have a caustic

interference, which form light and dark patches (Hill and McGraw 2006; Doucet and Meadows 2009). Iridescence can be caused by both interference and diffraction, and appears visually different depending on the viewing geometry (Doucet and Meadows 2009).

The majority of studies on avian plumage have focused on tristimulus colorimetric variables: hue, brightness, and chroma. Hue is typically the wavelength of the highest reflectance peak, brightness is the average reflectance across the avian-visual spectrum, and chroma is the difference between the maximum and minimum reflectance divided by the average reflectance (Hill and McGraw 2006). Some studies have examined each variable separately (e.g. Doucet et al. 2005; Siefferman and Hill 2005a; Siefferman and Hill 2005c), while other studies have combined all tristimulus colorimetric variables into single variables with principal component analysis (e.g. Doucet 2002; Bitton et al. 2008). Tristimulus analysis is an efficient method for analyzing plumage that does not vary in coloration. However, for birds with highly variable iridescent plumage (e.g. European starlings), it is best to average the reflectance into bins across wavelengths (10-20nm) where each bin counts as a variable within the spectrum (Cuthill et al. 1999; Hill and McGraw 2006; Meadows et al. 2011).

Plumage spectral characteristics can be an important attribute to assess male mates. For example, female zebra finches (*Taeniopygia guttata*) preferentially chose UV-brighter males in an experimental environment that contained UV-light, but appeared to randomly associate with males of different

UV-brightness levels in an environment lacking UV-light (Bennett et al. 1996). Johnsen et al. (1998) found that female common bluethroats (*Luscinia svecica*) preferred to mate with UV-brighter males (no sunblock treatment) over UV-duller males (sunblock treatment). Likewise, Nolan et al. (2010) found male and female king penguins (*Aptenodytes patagonicus*) (a sexually monomorphic bird) that received a sunblock treatment on their auricular patch took a longer time to find a partner in comparison to individuals that did not receive a sunblock treatment. Although these studies provide support that UV-light is important for assessing plumage quality, there could be a chance that the sunblock itself used in the studies may have deterred potential mates.

The good-genes hypothesis has been used to explain elaborate male plumage via female-driven mate choice (Andersson et al. 1998), but there is also evidence that bright female coloration and ornaments may have evolved via male mate choice in some species (Hill 1993; Griggio et al. 2009). Griggio et al. (2009) suggested that directional selection exists for ornamented females and may be associated with indirect genetic benefits. Many female passerine species display a cryptic plumage composed of dull and dark colors (Baker and Parker 1979), but some socially monogamous female passerine species have elaborate plumage (Griggio et al. 2005) (e.g. European starlings, wood warblers (Parulidae)). Female ornamentation is thought to result from only some of the sexually selected genes being expressed in females while always being expressed in males (Johnstone et al. 1996).

Female ornaments appear to play a role in male-driven mate choice. For instance, Hill (1993) found more reflective (brighter) female house finches (*Carpodacus mexicanus*) were preferred as mates as compared to less reflective (duller) females. Similarly, Amundsen et al. (1997) found that male bluethroats preferred to associate with females displaying a brighter blue throat-patch than with duller females. Griggio et al. (2005) found that female rock sparrows (*Petronia petronia*) with larger yellow breast patches were courted more by males than were females with experimentally reduced breast patches. These studies are supportive of mutual ornamentation evolving in some passerines because of its role in predicting quality of females. Brighter and more colorful female passerines have been found to have a greater realized reproductive success, fledgling success, and an earlier laying date than duller and less colorful females (e.g. Komdeur et al. 2005; Siefferman and Hill 2005b). Males mating with ornamented females may be gaining the indirect benefit of his offspring receiving the female's good genes that indirectly express these traits.

Structural plumage spectral characteristics have been used as a predictor for avian body (phenotypic) condition (Johnsen et al. 2003) and nutritional condition (measured by feather growth) (Keyser and Hill 1999; Doucet 2002). The formation of pigmented-based plumage is costly to birds as the cellular production of some pigments is metabolically demanding (Olson and Owens 1998). Highly ornamented plumage is also harder to maintain than less ornamented plumage (Walther and Clayton 2005). For example, iridescent

plumage in mallards (*Anas platyrhynchos*) was found to be less hydrophobic than non-iridescent plumage (Eliason and Shawkey 2011). The iridescence has a maintenance cost because the ornamented mallards have to care for the iridescent plumage more often to maintain its hydrophobicity in comparison to non-iridescent plumage. Doing so would decrease time spent on other activities, such as foraging, and vigilance (Walther and Clayton 2005). Therefore, high-quality iridescent plumage may honestly signal potential mates that he or she has the ability to avoid predators and forage effectively, while still maintaining plumage quality.

Structural plumage brightness has also been correlated with parental care. For example, Siefferman and Hill (2005a) found that more brightly colored male eastern bluebirds (*Sialia sialis*) provisioned to their social mate significantly more than did duller males. Ligon and Hill (2010) discovered that brighter UV-blue male eastern bluebirds provisioned nestlings more than duller males. Also, female eastern bluebirds given a large food supply were in better condition and displayed a brighter structural-blue plumage than females that had restricted food access (Siefferman and Hill 2005b). Brighter wild female eastern bluebirds were also found to provide more care to their young than duller wild females (Siefferman and Hill 2005b).

Recent studies have been analyzing plumage brightness and its relation with reproductive success (e.g. Siefferman and Hill 2003; Doucet et al. 2005; Komdeur et al 2005). Doucet et al. (2005) found that the brightness of the white

patch of male black-capped chickadees (*Poecile atricapillus*) and the UV-chroma of their melanin-based plumage were positively correlated with their realized reproductive success. Likewise, Siefferman and Hill (2003) found that brighter male eastern bluebirds fledged more offspring than did duller males. Brighter wild female eastern bluebirds were also hatched their first egg earlier and fledged more nestlings in comparison to duller females (Siefferman and Hill 2005b).

Fisher (1930) hypothesized that the sex ratio of animals should be stable at 1:1; however unity in sex ratios does not occur for most birds, and broods are typically either more male- (e.g. Sheldon et al. 1999; Ligon and Hill 2010) or female-biased (e.g. Ligon and Ligon 1990; Bradbury et al. 1997). Female Neognathid birds are heterogametic (ZW), while males are homogametic (ZZ) (Griffiths et al. 1998). Females could potentially control the sex of the ovum either during meiosis or through internal sex-specific egg mortality, such as embryo reabsorption, and hence alter the primary sex ratio (Ligon and Ligon 1990; Emlen 1997; Budden and Dickinson 2009). Reabsorption of the ovum may be a costly mechanism as females would then need to create a new ovum of the preferred sex. Sex ratio adjustments can also occur once the eggs hatch through sex-specific nestling mortality, resulting in secondary sex ratios that differ from 1:1 (e.g. Komdeur and Pen 2002; Budden and Dickinson 2009). Ligon and Hill (2010) found that female eastern bluebirds that paired with brighter males in comparison to duller males showed biased parental care towards sons as opposed to

daughters, which ultimately resulted in male offspring being in better condition at fledge than female offspring.

Mate choice is often invoked to explain sex allocation theory (Ellegren et al. 1996; Komdeur and Pen 2002). If a female bird mates with a male with good genes that are indirectly expressed through his phenotypic attractiveness, then her sons will inherit their father's good genes. Females that choose the most attractive males would effectively increase their own evolutionary fitness by rearing more sons than daughters because the attractive phenotypes are expressed more in males (Komdeur and Pen 2002). Female blue tits preferred to mate with males that had brighter UV-blue plumage; the brightest adult males had a male-biased offspring sex ratio in their social nest in comparison to duller UV-blue males (Sheldon et al. 1999). A male-biased sex ratio of a male's young (both within his nest and in those of others) could provide support for the hypothesis that plumage spectral characteristics can be used as a predictor of nestling sex ratios.

Hackle (throat feathers) length and iridescent coloration are characteristics used in mate choice in European starlings (Komdeur et al. 2005). Males display their purple and green iridescent hackles while singing to potential mates during courtship (Kessel 1957; Feare 1984). Bennett et al. (1997) found that female European starlings preferred to associate with males having saturated purple hackles over males having unsaturated green hackles. Komdeur et al. (2005) discovered that the hackles of European starlings were used in mutual mate

choice, became longer with age, and that age had a positive correlation with nestling fledging success.

European starlings are socially monogamous with the capacity to be facultatively polygynous (Pinxten and Eens 1997; Gwinner and Schwabl 2005). Starlings will engage in extra-pair copulations that often result in extra-pair young (Smith and Vonschantz 1993). European starlings have been found to exhibit a high number of successful extra-pair copulations (resulting in extra-pair young) in this breeding population (unpublished data) and in the populations of others (e.g. Pinxten et al. 1993). Similarly, females often mate outside their pair bond and then lay the egg either in her own nest or in the extra-pair male's nest (quasi-brood parasitism) (Pinxten et al. 1993). There are also instances of intraspecific brood parasitism (egg dumps), whereby a female will mate with an entirely different male and then lay the egg in the nest of a different pair (Pinxten et al. 1993). During this process, the female will dispose of one of the host pair's eggs.

European starlings are cavity nesters and lay one egg per day with clutch sizes averaging from four to five eggs (Feare 1984). The incubation period is 10-12 days after the last egg is laid, and the offspring remain in the nest for approximately 21 days whereupon they fledge (Feare 1984). The fledglings depend on their parents for approximately 12 days after leaving the nest (Feare 1984). Second broods can occur (depending on latitude of their breeding site), but there is a reduced number of active nests (Kessel 1957) and smaller clutch sizes (Feare 1984).

Male European starlings guard their social mate until laying is complete (Pinxten and Eens 1997). Males do not guard their extra-pair females (Pinxten et al. 1993). Extra-pair young care from their social father but not from their genetic father (Sandell et al. 1996).

This thesis examines if mate choice in European starlings is based upon plumage spectral characteristics signaling an adult male or female's genetic quality. I will investigate the role of hackle spectral characteristics on mate choice in European starlings by using principal components of hackle reflectance as a biological predictor of genetic quality. I will be correlating spectral characteristics with adult body condition, reproductive success, parental provisioning effort, and nestling sex ratio. I predict greater male and female body condition, provisioning effort, and reproductive success when adults exhibit high-quality hackle spectral characteristics (e.g. greater brightness and more purple coloration). I also predict that males and females with high-quality hackle spectral characteristics will produce more male offspring in comparison to those with low-quality hackle spectral characteristics.

Overall this study is designed to test the idea that genetic quality is associated with plumage signaling in mate choice. However, I am unable to specifically test the prediction that offspring produced by high-quality adults have increased survivorship (i.e. good genes). By understanding the effect that spectral characteristics of feathers have on mate choice in birds, variation in plumage amongst different avian populations can be better understood.

## **MATERIALS AND METHODS**

### **Study Site**

Field research was conducted on Saint Mary's University campus, in Halifax, Nova Scotia, Canada (44° 37' 54.07" N, 63° 34' 47.09"). A total of 40 nest boxes were erected on trees around the campus in 2007 with five more added in 2009. For this study, I collected data in 2011 and I also used data collected in 2007, 2008, and 2009. All necessary research permits were obtained from the Department of Natural Resources and Environment Canada. Animal care approval was provided by the Saint Mary's University Animal Care Committee.

Egg laying began on April 24<sup>th</sup> 2011; nests were checked daily from this date until all eggs had hatched. Nests were then left alone until nestlings were five days old (day 0 is hatch day). During the laying period, nests were always checked after 1100 hours because female European starlings typically lay their eggs between the hours of 0800 and 1100 (Feare 1984). These methodologies were consistent over all four years of the study.

A total of 18 broods was used for analysis in 2007 (adults = 33; nestlings = 55) (Worth-Kerr 2008; Wright 2008) 16 broods in 2008 (adults = 25; nestlings = 60) (Reeve 2009), 29 broods in 2009 (adults = 37; nestlings = 90)

(Wiebe 2010), and 30 broods in 2011 (adults = 39; nestlings = 124). Not all data from every individual was included in every analysis for this study (e.g. missing body measurements).

### **Adult and Nestling European Starlings**

Adults were captured using mo-traps (nestbox traps) (Stutchbury and Robertson 1986) when their nestlings were between five and thirteen days of age. Although nestlings are in the nest for up to 24 days (Feare 1984), we stopped approaching the nest and handling the nestlings when they were 14 days old to avoid having them fledge prematurely. Adult mass (to the nearest 0.1g) was measured using a Pesola spring scale. Tarsus length (to the nearest 0.01mm) of the right leg was measured five times with digital calipers and an average was calculated. Sex of adults was determined in the field by detecting a blue (male) or pink (female) skin coloration at the base of the beak and the absence (male) or presence (female) of a golden eye ring (Kessel 1951). Adult sex was confirmed through microsatellite DNA techniques. In addition, eight throat feathers (hackles) were plucked from each adult starling during each breeding season, and stored in a sealed and labeled plastic bag for spectrophotometric analysis.

Approximately 80-100  $\mu$ l of blood was extracted from the brachial vein of captured adults and 30-50  $\mu$ l of blood was extracted from the metatarsal vein of

nestlings when they were five or six days old. All blood was stored in 800  $\mu$ l of lysis buffer at  $-80^{\circ}\text{C}$  to prevent DNA degradation in 2011. Blood was stored in 95% ethanol at  $-20^{\circ}\text{C}$  in the 2007-2009 field seasons.

Adults were banded on their right tarsus with a Canadian Wildlife Service (CWS) band which was engraved with a numerical code specific to that individual. A yellow plastic band was placed above the male's CWS band to differentiate them from females, who had a pink band above their CWS band. A two-band unique color combination was applied to the left tarsus to help distinguish among individuals at the study site.

### **Provisioning**

One-hour provisioning watches were done between 0600 and 1100 hours AST when nestlings were 7-8 days and 13-14 days old, with the observers viewing with binoculars from 25-30 meters away. Starlings provision young at high rates during the morning (Kessel 1951; Feare 1984; Mennechez and Clergeau 2006) in comparison to the afternoon. Two-day windows were provided in the event of poor weather conditions. The one-hour period began with the arrival of a parent to the nestbox with food. The provisioning times and sex of the parents (determined by bands on tarsus) were recorded. The sexes of unbanded adults were determined by noting a blue (male) or pink (female) skin coloration at the base of the beak and glossy (male) or dull (female) plumage. However, if the

sex of unbanded adults could not be determined by the field researcher, then a general tally of provisioning visits was done, and the data were excluded from the analysis. The presence of food in the adult's bill was also noted. The provisioning watches were used to analyze adult male and female mean provisioning effort per nestling.

### **DNA Analysis**

DNA extraction, amplification (polymerase chain reaction (PCR)), multiplex testing, and genetic sexing were done at the Molecular Ecology and Evolution Lab at Saint Mary's University, Halifax, Nova Scotia. Multiplex imaging was done at the Bedford Institute of Oceanography, Dartmouth, Nova Scotia. For the 2007-2009 DNA protocols, please refer to Wright (2008), Worth-Marr (2008), Reeve (2009), and Wiebe (2010).

### *Digestion and Extraction*

The digestion and extraction process took five days for every set of samples. On the first day, raw blood samples were diluted to 5% blood concentration levels in new lysis buffer (400  $\mu$ l in total) and digested for one night. On the second day, 33.3  $\mu$ l of proteinase-K was added to each sample and digested overnight. On the third day, another 33.3  $\mu$ l of proteinase-K was added to each sample, and then the sample was placed into a 65°C water bath for an

hour, followed by another hour of incubation at 37°C. Once the incubation process was complete, a final 33.3 µl of proteinase-K was added to each sample and digested overnight at room temperature. On the fourth day, a mixture of phenol:chloroform was added twice to each sample to ensure that all protein and other waste products were dissolved. Samples were then purified with chloroform. After extraction, the DNA was precipitated out of solution using ice-cold 95% ethanol, and stored overnight in a -20°C freezer. On the fifth day, the precipitated DNA was pelleted, isolated, and stored in TE<sub>0.1</sub>. The amount of TE<sub>0.1</sub> used to dissolve the DNA varied depending on the size of the pellet present in the vial. If no pellet was present, only 200 µl of TE<sub>0.1</sub> was used. However, in most cases, 1000 µl of TE<sub>0.1</sub> needed to be used because of the high yield of DNA from the samples.

#### *DNA Quantity and Quality*

DNA quantity was digitally calculated using a Thermo Scientific NanoDrop 2000 (Fisher Scientific<sup>LTD</sup>, Ottawa, ON, Canada). Two DNA quantity (ng/µl) readings were averaged for each sample, and that value was used as the functional concentration. Five ng/µl working aliquots were made and gel electrophoresis was performed to confirm DNA quantity.

The 2% agarose gels were dyed with 8 µl of SYBR® Green (Applied Biosystems, Foster City, CA, USA); 20 µl of each sample were loaded into the wells of the gel. The brightness of each sample was compared against the

brightness of a Low DNA Mass™ Ladder (LDML) (Invitrogen™, Grand Island, NY, USA). If the brightness was similar for both, then the functional concentrations calculated on the NanoDrop were deemed accurate. However, if the bands were too bright or too dim, new dilutions were made until the brightness matched that of the LDML.

DNA quality was determined by using the same 2% agarose gels that were run to determine DNA quantity. Good quality DNA was above 2000 base pairs, which was represented by the top band of the LDML.

#### *Polymerase Chain Reaction (PCR)*

The concentration of DNA required for each PCR was determined mathematically. The haploid avian genome is approximately one third the size of the haploid human genome (Organ et al. 2007). One copy of the haploid human genome is  $3 \times 10^9$  base pairs (bp) and has a mass  $3.4 \times 10^{-3}$  ng (Cha and Thilly 1993); thus, one copy of the avian genome is  $1.13 \times 10^{-3}$  ng. To have an efficient PCR, there must be at least 1500-3000 copies of the region(s) of interest (Timothy Frasier, pers.comm.), therefore, approximately 3.4 ng of avian DNA is required for each PCR.

The PCR cocktail consisted of bovine serum albumin (BSA) (0.16 mg/μl), PCR buffer (1 x), dNTPs (0.2 mM), MgCl<sub>2</sub> (1.5 μM), *Taq* polymerase (0.05 U/μl), reagent water (autoclaved filtered water), and the desired primer's forward and reverse sequence reagent. Each primer was tested individually under a

temperature gradient of 50°C, 55°C, and 60°C using three different DNA samples from the 2011 field season. Each amplified locus was visualized using gel electrophoresis with a 2% agarose gel, and the most efficient annealing temperature was determined based on the primer's specificity to the locus of interest and the amount of DNA amplified.

Primers with a fluorescently tagged forward sequence for nine polymorphic loci were used in 2011 (Table 1). When excited by a laser using a 3500 xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), NED fluoresces yellow, PET fluoresces red, VIC fluoresces green, and 6-FAM fluoresces blue. Each reaction consisted of 3.4 µl of 1 ng/µl DNA and 16.6 µl of PCR cocktail. Only the first six loci were used for parentage analysis because Sta213 had a mutation, SS3-42C had abnormal peak patterns, and SS1-11 amplified poorly.

The amplified DNA for each desired annealing temperature was then tested in the capillary electrophoresis machine using 2 µl of amplified DNA, and 8 µl of a cocktail containing formamide and a DNA size standard (GeneScan™ 600 LIZ® Size Standard (Applied Biosystems, Foster City, CA, USA)). The loci were then visualized using GeneMarker™ 2.2.0 software (SoftGenetics LLC™, State College, Pennsylvania, USA), and the concentration of each primer needed for the multiplex reactions was determined by the size of the fluorescent peaks produced by each primer. Primer concentration was increased or reduced in order to achieve peak sizes around the range of 3000 maximums. All primers

except Sta70, SS3-42C, and SS1-11 were in H-W equilibrium and amplified between 6-14 different alleles.

The primers SS2-71B, ASE-18, SS3-42C, and SS1-6 were all used in 2007-2009 and 2011 (Tables 2-4) (Worth-Marr 2008; Wright 2008; Reeve 2009; Wiebe 2010). Additional primers used were: Sta269 in 2011; Sta213 and Sta70 in 2008, 2009, and 2011; SS1-11 in 2007 and 2011; Sta308 in 2008 and 2011; Sta317 in 2008; FhU-3, and Pca-7 in 2007. Finally, all loci from 2007-2009 were visualized on agarose gels and scored by hand, whereas in 2011 the loci were visualized using GeneMarker™ 2.2.0 Software (SoftGenetics LLC™, State College, Pennsylvania, USA).

### *Multiplex Reactions*

The multiplex reaction scenarios were chosen based on the primers' annealing temperatures, fluorescent tag, and target-sequence size (bp). Various multiplex reaction scenarios were tested using a 3500 xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The most efficient multiplex reactions used to genotype the 2011 starling breeding population consisted of reaction 1 (58°C Annealing Temperature) which had Sta70 (NED), Sta269 (VIC), ASE-18 (6FAM), and SS1-6 (PET), and reaction 2 (53°C Annealing Temperature) which had Sta308 (VIC), SS2-71B (NED), and SS3-42C (6FAM).

### *Genotyping*

Multiplex imaging was performed using a 3130 xl Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Bedford Institute of Oceanography. Gene scoring in 2011 was done using GeneMarker™ 2.2.0 Software (SoftGenetics LLC™, State College, Pennsylvania, USA). Primer SS3-42C was deemed unusable because it produced irregular peak patterns on the electropherogram, and was therefore omitted from data analysis.

### *Sex Ratio Analysis*

The sexes of all individuals (adults and five day-old nestlings) from 2007 and 2011 were determined using P2 and P8 primers that target two CHD (chromo-helicase-DNA binding) genes on the W and Z chromosomes located in the Neognathid genome (Griffiths et al. 1998). The P2 and P8 primers amplify homologous loci on both the CHD-W and CHD-Z genes. Amplified DNA was analyzed via gel electrophoresis and resulted in two bands appearing for females and one band appearing for males. The sexes were determined by Worth-Marr (2008) and by Ouedraogo (2012). Primary sex ratio calculations used in this study are of my own.

## **Parentage Assignment**

Maternity (within-pair, extra-pair, and brood-parasitic young) and paternity (within-pair, extra-pair, and brood parasitic young) were determined for the 2007 and 2011 breeding years and were assigned genetic parents using Cervus 3.03 software (Field Genetics, London, UK) (Kalinowski et al. 2007). We could not assign parentage to the extra-pair and brood-parasitic young in 2008 and 2009 because in these two years, the loci were scored by hand and contained a high number of mismatches. However, the proportion of within-pair young produced was determined for all four years (2007-2009, 2011).

## **Hackle Color Analysis**

Eight hackles from adult male and female European starlings were each mounted separately on a labeled piece of black matted Bristol board (e.g. Bitton et al. 2008). Each of the eight hackles were analyzed separately due to the color variation across individual feathers (Meadows et al. 2011). Hackle spectra were examined using a USB4000 spectrophotometer (Ocean Optics, Dunedin, FL, USA) and a QR400-7-UV-VIS reflectance probe (Ocean Optics, Dunedin, FL, USA) with a PX-2 pulsed xenon-light source (Ocean Optics, Dunedin, FL, USA). The ultraviolet-visible 4mm probe (QR400-7-UV-VIS) was inserted into a PX-2 pulsed xenon-light source through a single optical path, with the other connector-end placed into a USB4000 Spectrophotometer (Ocean Optics, Dunedin, FL,

USA). The probe was held 3mm above a feather using an Erlenmeyer flask rubber stopper as the probe holder to maintain a consistent distance. OOIBase software (Ocean Optics, Dunedin, FL, USA) was used to record the reflectance data of each feather. The spectrophotometer was calibrated using a Spectralon white reflectance standard (Labsphere, Ottawa, ON, Canada) and set with an integration time between 55 and 65 msec to achieve peak maximums of 50 000 counts. The software was programmed to take an average of 30 sequential readings per feather measurement with a moving-average-window (boxcar) of five. Two measurements were taken and averaged for each hackle.

## **Statistical Analyses**

### *Hackle Spectral Data*

The hackle spectral data were analyzed (2007-2009, 2011) at the University of Windsor, Ontario, Canada. Mean percent reflectance and standard error values were calculated per male and female in 20nm bins between 300-700nm (avian-visual range (Hill and McGraw 2006)). Starling hackles displayed multiple peaks along the avian-visual spectrum (e.g. Figure 3), so standard colorimetric variables (e.g. brightness, chroma, and hue) could not be used to statistically analyze the reflectance data (Cuthill et al. 1999; Meadows et al. 2011). The 20nm bins represent correlated variables along each individual's mean hackle spectrum. Brightness is thought to be the largest correlate between

each 20nm of the percent reflectance data (Cuthill et al. 1999). I did not correct for brightness in this study as it could be a plumage component that starlings use to assess mate quality (Stéphanie Doucet, pers. comm.). Principal components analysis (PCA) was used to transform the reflectance data into single variables that explained spectral variation for each adult male and female. A total of 20 principal components (PC1-PC20) were calculated separately for adult male and female European starlings. PC4 to PC20 for males and PC3 to PC20 for females were not deemed interpretable because their eigenvalues were below the accepted value of 1.00 (e.g. Hill and McGraw 2006) (Tables 5 and 6).

#### *Adult Condition*

Data from male and female European starlings were analyzed separately as they varied significantly in mass (g) (males =  $85.2 \pm 0.56$ ; females =  $80.2 \pm 0.56$ ) (unpaired  $t_{134} = 6.21$ ,  $P < 0.0001$ ) and tarsus length (mm) (males =  $35.1 \pm 0.12$ ; females =  $34.4 \pm 0.12$ ) (Mann-Whitney  $U = 1397$ ,  $n_1 = 57$  (males),  $n_2 = 79$  (females),  $P = 0.0002$ ). Least squares regressions were used to analyze male and female mass in relation to their tarsus length (males:  $y = 1.587x + 29.51$ ; females:  $y = 2.707x - 12.91$ ) (e.g. Pärt 1990; Labocha and Hayes 2012). The residuals were then used as a metric of condition for each adult. A positive value indicated an adult in good condition while a negative value indicated an adult in poor condition.

### *Provisioning Effort*

Provisioning effort per nestling was determined by taking the total number of nest visits an adult male or female made during the hour and dividing it by the total number of offspring present during the provisioning watch. A mean total provisioning effort per nestling was calculated for adult males and females by averaging the total provisioning effort per nestling values on day 7/8 and day 13/14.

### *Reproductive Success*

Cervus 3.03 software (Field Genetics, London, UK) (Kalinowski et al. 2007) was used to determine the realized reproductive success of males and females. The 2007 and 2011 genetic data were the only years deemed useable to calculate the total number of genetically-related young produced. However, the 2007-2009, and 2011 genetic data were used to calculate the proportion of within-pair young in a brood by counting the total number of five day-old young that a male or female genetically produced in their own nest and dividing it by the total number of young present (the other nestlings would be extra-pair or brood-parasitic young).

### *Sex Allocation*

The number of male and female offspring that an adult genetically produced was analyzed for 2007 and 2011. Proportions were calculated by

dividing the total number of genetically-related male offspring by the total number of genetically-related offspring an adult produced overall. The proportion of male young produced was used as a variable to test for potentially biased nestling sex ratios between adult males and females.

### *Statistical Testing*

Statistical analyses were performed using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA). The data were checked for normality by using the D'Agostino and Pearson omnibus normality test. Hackle principal components (PCs) were plotted against adult body condition, provisioning effort, reproductive success, and nestling sex ratios. All correlative tests were two-tailed and probability values were considered statistically significant at the  $\alpha \leq 0.05$  level.

**Table 1:** Characteristics of nine polymorphic loci of European starlings used in 2011 as described in Loyau et al. (2005), Rubenstein (2005), and Celis et al. (2007).

	Primer Sequence (5'-3') with dye labels	Ta (°C)	No. of Alleles	H <sub>o</sub>	H <sub>e</sub>	HWE
<b>SS2-71B</b>	F-NED-CACACCCAACATGTAACAAATAATCTTACA	55	9	0.689	0.688	NS
	R-CTTTGAGCCTCTGCTTTTAGAAATTG					
<b>ASE-18</b>	F-6FAM-ATCCAGTCTTCGCAAAGCC	60	12	0.813	0.823	NS
	R-TGCCCCAGAGGGAAGAAG					
<b>SS3-42C</b>	F-6FAM-TATATCCCAGGGAGGGTTGTGGTGTG	ND	ND	ND	ND	ND
	R-ATCAAAGTGCAGCAGGACTCTGACTGTG					
<b>SS1-6</b>	F-PET-TTTCAGTGGCTGGATCTGGTAAAC	55	6	0.746	0.729	NS
	R-CTAGCAACATATAGCCCAAGCTGTATTGAT					
<b>Sta213</b>	F-PET-TTGGCCTTGCTGAACCTCTT	ND	ND	ND	ND	ND
	R-GATCAAGTGCACCTTCAGCA					
<b>Sta70</b>	F-NED-AGGTGTGTGGGAGAGAATGG	60	14	0.578	0.878	*
	R-ATGGACAAAAGAAGGCATGG					
<b>Sta269</b>	F-VIC-TGGGGATTAATAGGGGTGTG	60	8	0.766	0.793	NS
	R-GCAGTGAGAAGAGGGCTTTG					
<b>SS1-11</b>	F-NED-AAATTTGAACCGATCCAGCCTGTTA	ND	ND	ND	ND	ND
	R-CTCGCTCCCTCTCCCTCTTTCAC					
<b>Sta308</b>	F-VIC-GCTTAAGCACCCCTCACAAC	55	9	0.930	0.888	NS
	R-CTGCAATCAGGGTTTGATT					

\*Not in Hardy-Weinberg Equilibrium (HWE)

ND: Not Determined

NS: In HWE.

**Table 2:** Characteristics of six polymorphic loci of European starlings used by Wiebe (2010) as described in Loyau et al. (2005), Rubenstein (2005), and Celis et al. (2007).

	Primer Sequence (5'-3')	Ta (°C)	No. of Alleles	H <sub>o</sub>	H <sub>E</sub>	HWE
<b>SS2-71B</b>	F-CACACCCAACATGTAACAAATAATCTTACA	63	12	0.560	0.852	*
	R-CTTTGAGCCTCTGCTTTTAGAAATTG					
<b>ASE-18</b>	F-ATCCAGTCTTCGAAAAGCC	57	28	0.817	0.903	ND
	R-TGCCCCAGAGGGAAGAAG					
<b>SS3-42C</b>	F-TATATCCCAGGGAGGGTTGTGGTGTG	60	13	0.769	0.875	ND
	R-ATCAAAGTGCAGCAGGACTCTGACTGTG					
<b>SS1-6</b>	F-TTTCAGTGGCTGGATCTGGTAAAC	59	19	0.541	0.833	*
	R-CTAGCAACATATAGCCCAAGCTGTATTGAT					
<b>Sta213</b>	F-TTGGCCTTGCTGAACCTTCTT	59	33	0.794	0.945	ND
	R-GATCAAGTGCACCTTCAGCA					
<b>Sta70</b>	F-AGGTGTGTGGGAGAGAATGG	62	24	0.509	0.945	ND
	R-ATGGACAAAAGAAGGCATGG					

\*Not in Hardy-Weinberg Equilibrium (HWE)

ND: Not Determined

NS: In HWE.

**Table 3:** Characteristics of eight polymorphic loci of European starlings used by Reeve (2009) as described in Loyau et al. (2005), Rubenstein (2005), and Celis et al. (2007).

	Primer Sequence (5'-3')	Ta (°C)	No. of Alleles	H <sub>o</sub>	H <sub>e</sub>	HWE
<b>SS2-71B</b>	F-CACACCCAACATGTAACAAATAATCTTACA	63	7	0.590	0.752	NS
	R-CTTTGAGCCTCTGCTTTTAGAAATTG					
<b>ASE-18</b>	F-ATCCAGTCTTCGCAAAAGCC	57	10	0.730	0.790	NS
	R-TGCCCCAGAGGGAAGAAG					
<b>SS3-42C</b>	F-TATATCCCAGGGAGGGTTGTGGTGTG	60	13	0.660	0.780	NS
	R-ATCAAAGTGCAGCAGGACTCTGACTGTG					
<b>SS1-6</b>	F-TTTCAGTGGCTGGATCTGGTAAAAC	59	7	0.590	0.720	*
	R-CTAGCAACATATAGCCCAAGCTGTATTGAT					
<b>Sta213</b>	F-TTGGCCTTGCTGAACTTCTT	61	9	0.920	0.870	ND
	R-GATCAAGTGCACCTTCAGCA					
<b>Sta70</b>	F-AGGTGTGTGGGAGAGAATGG	62	7	0.870	0.750	ND
	R-ATGGACAAAAGAAGGCATGG					
<b>Sta308</b>	F-GCTTAAGCACCCCTCACAAC	60	10	0.940	1.00	ND
	R-CTGCAATCAGGGTTTGATT					
<b>Sta317</b>	F-TCGGGGACAGCAGGTATATT	59	6	0.780	0.870	ND
	R-ATGCTTCTCTCAGCAGCTC					

\*Not in Hardy-Weinberg Equilibrium (HWE)

ND: Not Determined

NS: In HWE.

**Table 4:** Characteristics of seven polymorphic loci of European starlings used by Worth-Kerr (2008) and Wright (2008) as described in Loyau et al. (2005) and Rubenstein (2005).

	Primer Sequence (5'-3')	Ta (°C)	No. of Alleles	H <sub>o</sub>	H <sub>E</sub>	HWE
SS2-71B	F-CACACCCAACATGTAACAAATAATCTTACA	63	12	0.588	0.752	NS
	R-CITTTGAGCCTCTGCTTTTAGAAATTG					
ASE-18	F-ATCCAGTCTTCGCAAAAGCC	57	10	0.725	0.792	NS
	R-TGCCCCAGAGGGAAGAAG					
SS3-42C	F-TATATCCCAGGGAGGTTGTGGTGTG	60	13	0.663	0.779	NS
	R-ATCAAACGCAGCAGGACTCTGACTGTG					
SS1-6	F-TTTCAGTGGCTGGATCTGGTAAAC	59	7	0.593	0.720	*
	R-CTAGCAACATATAGCCCAAGCTGTATTGAT					
SS1-11	F-AAATTTGAACCGATCCAGCCTGTTTA	61	10	0.840	0.851	ND
	R-CTCGCTCCCTCTCCCTCTTTCAC					
FhU-3	F-ATATTTCCCATAGATAATGG	49	9	0.250	0.768	*
	R-ATAGTGTGTCTTAAGGTCTCT					
Pca-7	F-TGAGCATCGTAGCCAGCAG	55	6	0.221	0.433	ND
	R-GGTTCCAGGACACCGTCACAATG					

\*Not in Hardy-Weinberg Equilibrium (HWE)

ND: Not Determined

NS: In HWE.

**Table 5:** Eigenvalues and percent of variation explained for PC1-PC20 from male Euroj

<b>Principal Component</b>	<b>Eigenvalue</b>	<b>Percent of Variation Explained</b>
PC1	15.06	71.72
PC2	3.57	17.00
PC3	1.73	8.25
PC4-PC20	--	3.03
<b>Total</b>	--	100.00

**Table 6:** Eigenvalues and percent of variation explained for PC1-PC20 from female Eur

<b>Principal Component</b>	<b>Eigenvalue</b>	<b>Percent of Variation Explained</b>
<b>PC1</b>	18.05	85.93
<b>PC2</b>	1.68	7.99
<b>PC3-PC20</b>	--	6.08
<b>Total</b>	--	100.00

## **RESULTS**

### **Hackle Spectrophotometry**

PC1 showed the least amount of variation in eigenvectors along the avian-visual spectrum for both sexes, and the eigenvector factor loadings were positive (Figure 1). However, the eigenvector factor loadings for PC2 (for males and females) and PC3 (for males only) varied substantially (Figures 1 and 2).

Male and female hackle spectral characteristics varied both in peak height and peak number among individuals. Figures 3, 4, and 5 illustrate the reflectance curves of six males, three males with the highest and three with the lowest PC1, PC2, and PC3 scores respectively. Likewise, Figures 6 and 7 illustrate the reflectance curves of six females, three having the highest and three with the lowest PC1 and PC2 scores respectively. These curves were used in order to understand how varying PC scores resulted in various hackle spectra shapes and intensities. The variation in PC1 scores was ultimately due to the variation in the mean level of percent reflection per individual. Adults with the lowest PC1 scores had the lowest percent reflectance in their hackle spectral curves while adults with the highest PC1 scores had the highest percent reflectance in their hackle spectral curves (Figures 3 and 6). Males and females with hackle spectra peaking trimodally in the UV (300-400nm), blue (450-490nm), and red wavelengths (620-700nm) had the lowest PC2 scores with their hackles having a purple appearance to humans. Males and females with hackle spectra peaking

bimodally in the UV and green-orange (500-620nm) wavelengths had the highest PC2 scores with their hackles having a green appearance to humans (Bennett et al. 1997; Komdeur et al. 2005). Males with the three highest and three lowest PC3 scores all had varying peak wavelengths and there was no apparent pattern among these individuals (Figure 5).

To summarize, PC1 explains variation in hackle brightness, PC2 explains variation in hackle color, and PC3 does not appear to explain any obvious variation in hackle spectral characteristics. PC1 will therefore be called “brightness”, and PC2 will be called “color”, with high PC2 scores being called “unsaturated green” and low PC2 scores being called “saturated purple” (Bennett et al. 1997; Komdeur et al. 2005). PC3 will remain as PC3.

### **Body Condition**

There was no significant correlation between male body condition and male hackle brightness (PC1 scores) ( $r = -0.15$ ,  $n = 54$ ,  $P = 0.28$ ) or hackle PC3 scores ( $r = 0.04$ ,  $n = 54$ ,  $P = 0.77$ ). There was a significant negative correlation between male hackle color (PC2 scores) and their body condition ( $r = -0.34$ ,  $n = 54$ ,  $P = 0.01$ ); males with purpler hackles had a significantly better body condition than did males with greener ones (Figure 8).

There was a significant positive correlation between female hackle brightness and condition ( $r = 0.47$ ,  $n = 72$ ,  $P < 0.0001$ ) (Figure 9). However, no

significant correlation existed between female hackle color and female condition ( $r = -0.08$ ,  $n = 72$ ,  $P = 0.52$ ).

### **Provisioning Effort**

There was a significant positive correlation between adult total mean provisioning effort per nestling by adult males and male hackle brightness ( $r_s = 0.39$ ,  $n = 32$ ,  $P = 0.03$ ) (Figure 10). No significant correlation existed between total mean provisioning effort and either male hackle color ( $r_s = -0.02$ ,  $n = 32$ ,  $P = 0.96$ ) or male hackle PC3 scores ( $r_s = -0.29$ ,  $n = 32$ ,  $P = 0.42$ ).

Adult female mean provisioning effort per nestling was not correlated with female hackle brightness ( $r_s = -0.11$ ,  $n = 38$ ,  $P = 0.49$ ) or with hackle color ( $r_s = 0.07$ ,  $n = 38$ ,  $P = 0.70$ ).

### **Reproductive Success**

There was no significant correlation between male realized reproductive success (2007 and 2011) and male hackle brightness ( $r_s = -0.22$ ,  $n = 27$ ,  $P = 0.26$ ), hackle color ( $r_s = -0.16$ ,  $n = 27$ ,  $P = 0.44$ ), or hackle PC3 scores ( $r_s = -0.17$ ,  $n = 27$ ,  $P = 0.37$ ).

There was also no significant correlation between the proportion of within-pair offspring that a male genetically produced during a breeding season and his

hackle brightness ( $r_s = -0.17$ ,  $n = 50$ ,  $P = 0.25$ ), hackle color ( $r_s = 0.09$ ,  $n = 50$ ,  $P = 0.54$ ), or hackle PC3 scores ( $r_s = 0.08$ ,  $n = 50$ ,  $P = 0.60$ ).

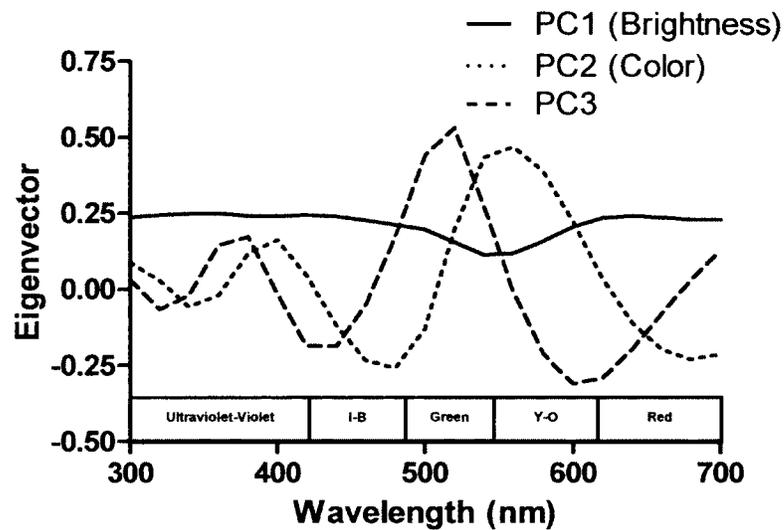
There was also no significant correlation between realized female reproductive success (2007 and 2011) and hackle brightness ( $r = 0.13$ ,  $n = 33$ ,  $P = 0.45$ ). However, female reproductive success was significantly correlated with hackle color ( $r = -0.38$ ,  $n = 33$ ,  $P = 0.03$ ) with females having purpler hackles producing more genetic young (Figure 11).

There was no significant correlation between the proportion of within-pair offspring that a female genetically produced during a breeding season and her hackle brightness ( $r_s = 0.21$ ,  $n = 56$ ,  $P = 0.11$ ) or hackle color ( $r_s = -0.15$ ,  $n = 56$ ,  $P = 0.26$ ).

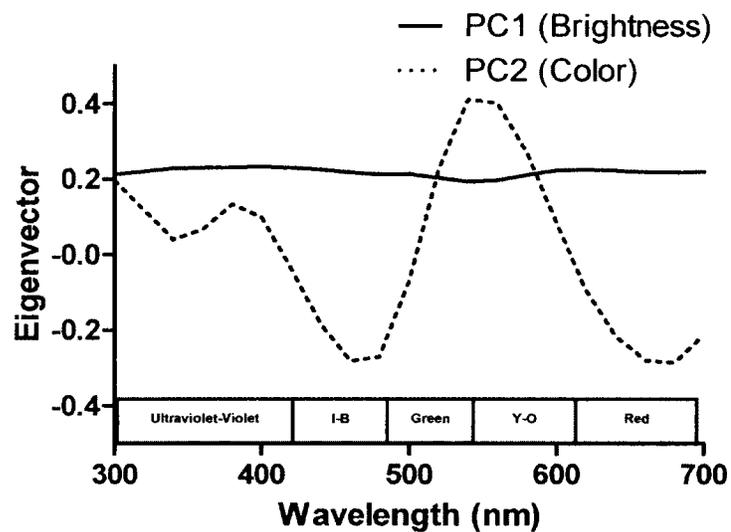
### **Sex Allocation**

The proportion of male offspring that a male sired in his own nest and in the nests of other females during the 2007 and 2011 breeding season was significantly correlated with male hackle brightness ( $r = 0.48$ ,  $n = 24$ ,  $P = 0.02$ ) (Figure 12), but not with hackle color ( $r = -0.19$ ,  $n = 24$ ,  $P = 0.38$ ) or PC3 scores ( $r = -0.18$ ,  $n = 24$ ,  $P = 0.40$ ).

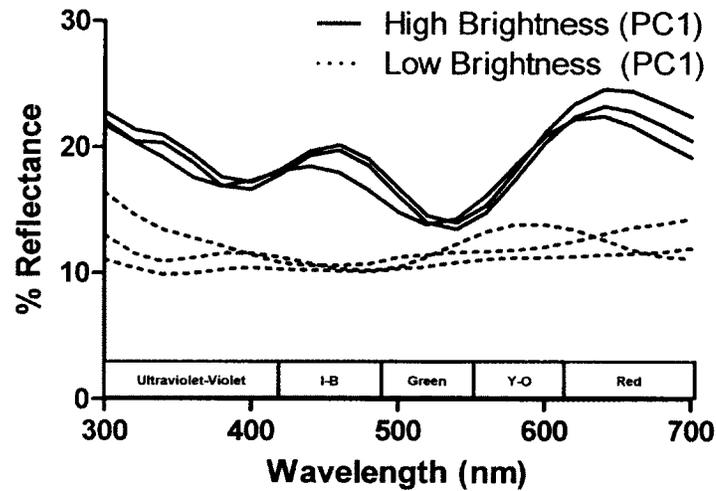
There was no significant correlation between the proportion of male offspring that a female produced in her own nest and in the those of other males during the 2007 and 2011 breeding season and female hackle brightness ( $r = -0.06$ ,  $n = 29$ ,  $P = 0.74$ ) or hackle color ( $r = 0.07$ ,  $n = 29$ ,  $P = 0.73$ ).



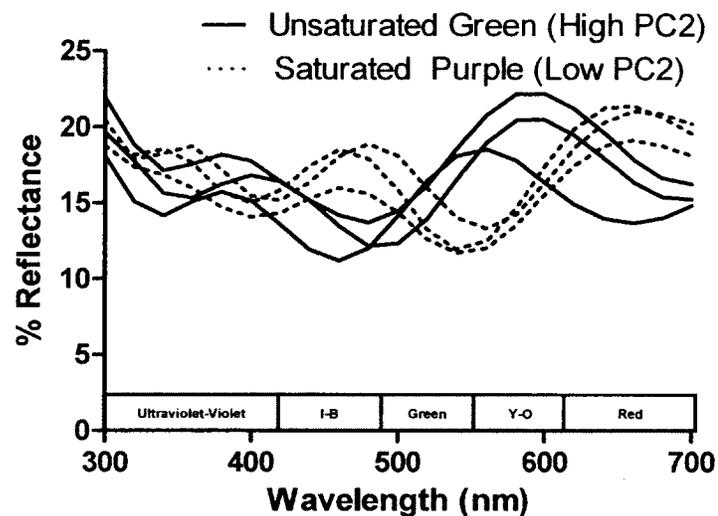
**Figure 1:** Eigenvectors of the first three PCs (brightness, color, and PC3) from male European starling hackle reflectance spectral data. The solid line denotes the brightness component (PC1), the dotted line denotes the color component (PC2) and the dashed line denotes PC3. The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.



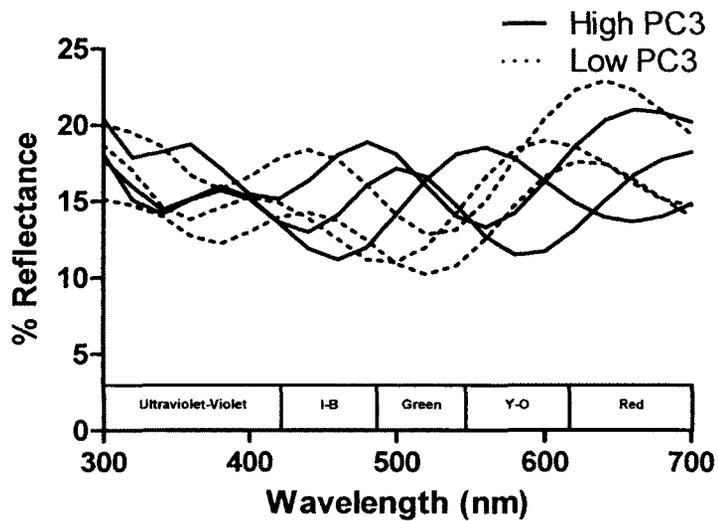
**Figure 2:** Eigenvectors of the first two PCs (brightness and color) from female European starling hackle spectral data. The solid line denotes the brightness component (PC1) and the dotted line denotes the color component (PC2). The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.



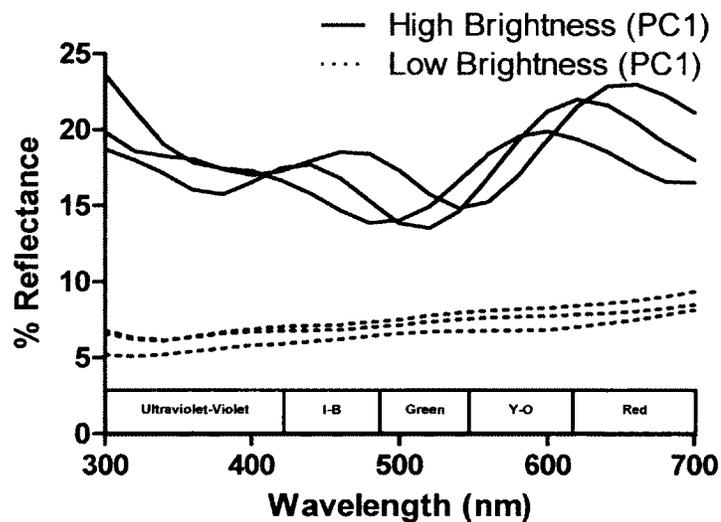
**Figure 3:** Example of male hackle spectra data from six separate European starling males. The solid line denotes three sampled males with the highest brightness (PC1 scores) and the dotted line denotes three sampled males with the lowest brightness. The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.



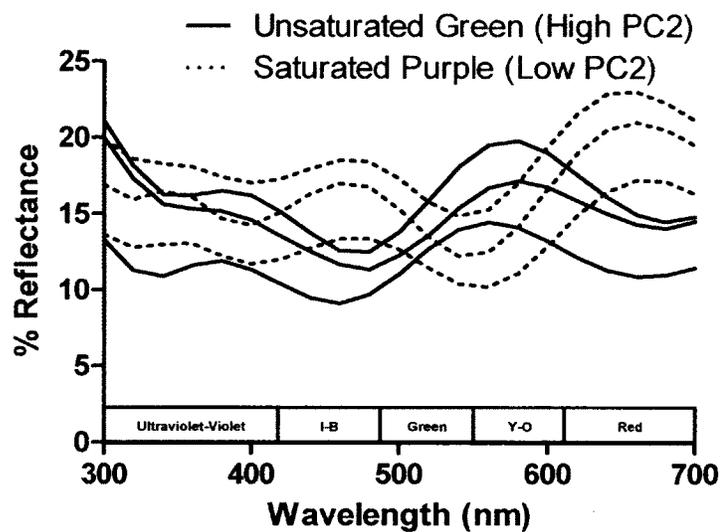
**Figure 4:** Example of male hackle spectra data from six separate European starling males. The solid line denotes three males with unsaturated green hackles (high PC2 scores) that peaked in the UV and green-orange wavelengths. The dotted line denotes three males with saturated purple hackles (low PC2 scores) that peaked in the UV-violet, indigo-blue, and red wavelengths. The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.



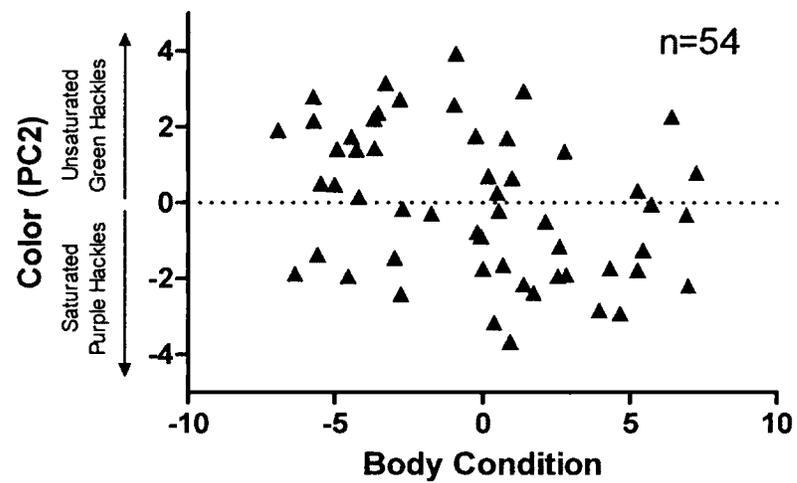
**Figure 5:** Example of six male hackle spectra from six separate European starling males. The solid line denotes three males with the highest PC3 scores and dotted line denotes three males with the lowest PC3 scores. The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.



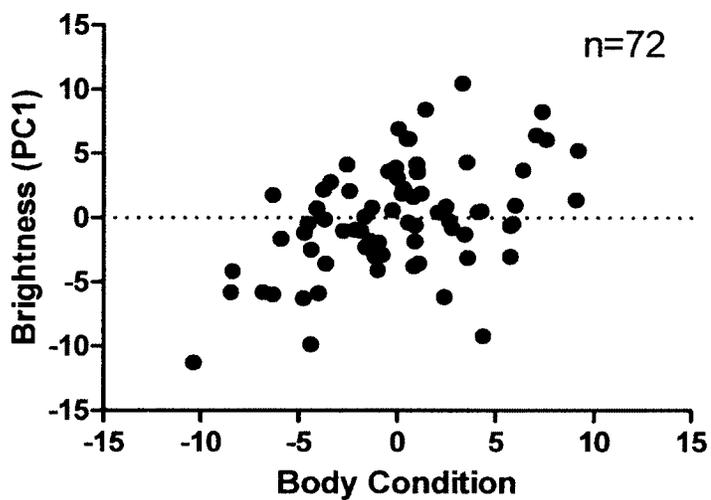
**Figure 6:** Example of female hackle spectra data from six separate European starling females. The solid line denotes three sampled females with the highest brightness (PC1 scores) and the dotted line denotes three sampled females with the lowest brightness. The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.



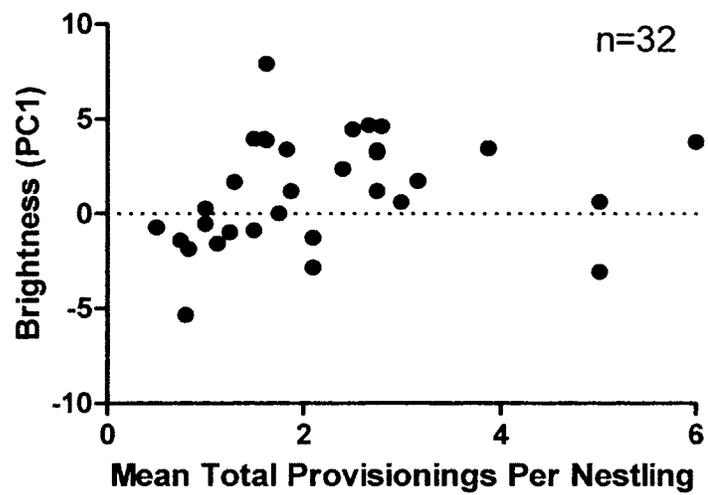
**Figure 7:** Example of female hackle spectra data from six separate European starling females. The solid line denotes three females with unsaturated green hackles (high PC2 scores) that peaked in the UV and green-orange wavelengths. The dotted line denotes three females with saturated purple hackles (low PC2 scores) that peaked in the UV-violet, indigo-blue, and red wavelengths. The x-axis includes color descriptors of where one would approximately expect the ultraviolet-violet, indigo-blue (I-B), green, yellow-orange (Y-O), and red color wavelengths to fall within.



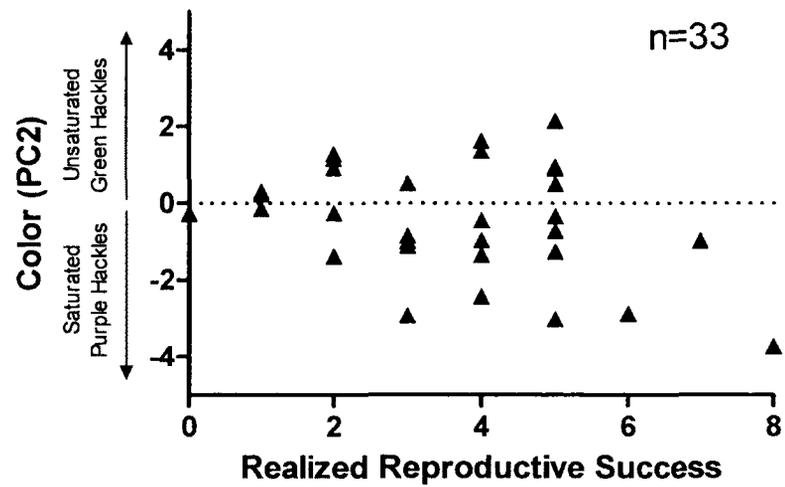
**Figure 8:** Hackle color (PC2 scores) of male European starlings plotted against male body condition (residuals of mass against tarsus length).



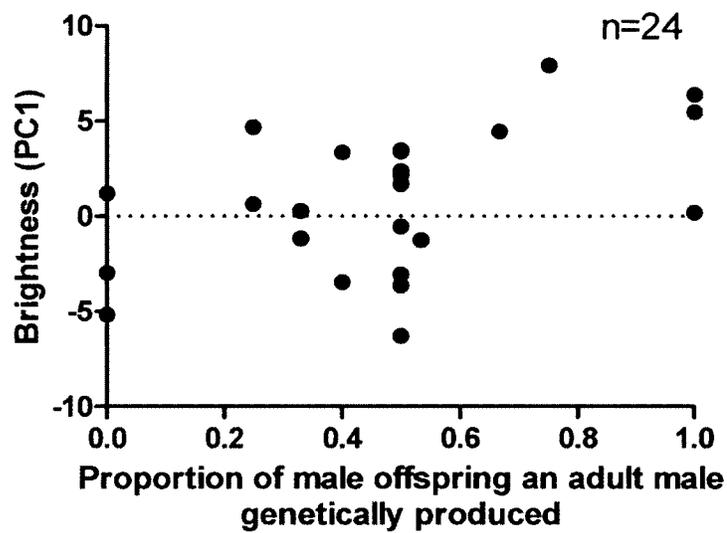
**Figure 9:** Hackle brightness (PC1 scores) of female European starlings plotted against female body condition (residuals of mass against tarsus length).



**Figure 10:** Hackle brightness (PC1 scores) of male European starlings plotted against the mean total provisionings per nestling.



**Figure 11:** Hackle color (PC2 scores) of female European starlings plotted against female realized reproductive success.



**Figure 12:** Hackle brightness (PC1 scores) of male European starlings plotted against the proportion of male offspring an adult male genetically produced during the breeding seasons (2007 and 2011 combined).

## **DISCUSSION**

### **Hackle Spectral Characteristics**

PC1 eigenvector factor loadings were relatively consistent for both males and females, and were positive throughout the avian-visual spectrum (300-700nm), suggesting that PC1 represents a brightness component (e.g. Endler 1990; Hill et al. 2005). The hackle spectral shapes for unsaturated green and saturated purple feathers were comparable to previous studies that analyzed European starling hackle spectral characteristics (Cuthill et al. 1999; Bennet et al. 1997). Cuthill et al. (1999) found, as did I, that both males and females had hackle reflectance curves peaking in either the UV (300-400nm), blue (450-490nm), and red (620-700nm) spectral ranges, or in the UV and green-orange (500-620nm) spectral ranges. Bennett et al. (1997) found the same spectral curve shapes in their study and noted that female European starlings preferred to associate with males that had hackles peaking in the UV, blue and red spectral wavelength ranges (appearing saturated purple to humans), which were represented by low PC2 scores in my study. Females did not prefer to associate with males that had hackles peaking in the UV and yellow-orange range (appearing unsaturated green to humans), which were represented by high PC2 scores in my study.

Cuthill et al. (1999) controlled for brightness in their study by subtracting the total mean brightness from every 20nm bin of spectral data for each

individual. My methodology included the brightness component because it is believed to be important in mate choice (e.g. Doucet and Montgomerie 2003).

### **Body Condition**

Males with saturated purple hackles were in significantly better body condition (residuals of mass against tarsus length) than males with unsaturated green hackles. Hackle brightness was not associated with condition in males. Male European starlings in this breeding population appear to signal their body condition through structural plumage coloration instead of brightness. Budden and Dickinson (2009) found that male western bluebirds (*Sialia mexicana*) with brighter structural-blue head plumage were in better body condition than males with duller head plumage. Similarly, Keyser and Hill (1999) found that the brightness of structural plumage in male blue grosbeaks (*Guiraca caerulea*) was positively correlated with their wing chord length (mm). They also found that male blue grosbeaks with high blueness scores (a metric that incorporates plumage brightness) were in better nutritional condition (tail feather growth rate (mm/week) over a breeding season) than males with low blueness scores (Keyser and Hill 2000). Nutritional condition (tail feather growth rate) was also positively and significantly correlated with PC1 scores (brightness, intensity, contrast, and peak reflectance) in male blue-black grassquits (*Volatinia jacarina*) (Doucet 2002). Murphy and Pham (2012) discovered that the brightness of structural plumage in turquoise-browed motmots (*Eumomota superciliosa*) was positively correlated

with mean tail feather growth (nutritional condition) for males but not for females. Lastly, body condition in adult males was not correlated with PC3 scores, and no results in my study support that PC3 is a biologically important variable (see Results).

Parasite abundance and immunosuppressant chemicals have been shown to reflect a bird's body condition in species such as house sparrows (Navarro et al. 2003), house martins (*Delichon urbica*) (Christe et al. 1998), and barn swallows (*Hirundo rustica*) (Saino et al. 1997). Hill et al. (2005) found male wild turkeys (*Meleagris gallopavo*) with high PC2 scores (redder males in their study) had a higher parasite load than males with low PC2 scores (bluer males in their study), but they did not compare plumage color to body condition or reproductive success. However, Bennett et al. (1997) found that adult female European starlings spent more time with males that had purpler hackles and less time with those having greener hackles. Possibly iridescent purple hackles in males are condition-dependent or age-dependent and act as honest indicators of male health and/or viability (i.e. good genes), evolving by parasite-mediated sexual selection (Hamilton and Zuk 1982). Females that are socially paired with males exhibiting greener hackles may obtain good genes for their offspring by acquiring extra-pair fertilizations from males with purpler hackles. I have not tested this possibility, but it remains a promising future research avenue.

Unlike males, adult female hackle brightness was positively correlated with female body condition. This finding has been documented in European rollers

(*Coracias garrulus*) where female plumage brightness was positively correlated with female body mass (Møller and Birkhead 1994). Likewise, female red-winged blackbirds that increased their epaulet brightness from one season to the next had a higher body mass and wing area in comparison to returning females that did not enhance their epaulet brightness over this time frame (Mountjoy and Lemon 1996). Amundsen et al. (1997) found that more colorful female common bluethroats were preferred by males over drabber ones, and that they were in better body condition than the duller females. Hackle brightness signals individual female body condition in our European starling population, but the data also suggests that purple-hackled females have a higher realized reproductive success than females with greener hackles. Overall, my results indicate hackle brightness in females is condition-dependent and could signal female health (i.e. genetic quality).

### **Provisioning Effort**

The total mean provisioning effort by adult males per nestling was positively correlated with their hackle brightness but not with their hackle color or PC3 scores. Plumage brightness has been correlated with provisioning effort in other species as well. Siefferman and Hill (2003) found that male eastern bluebirds with high plumage brightness and chroma made more visits to nestlings than males with low plumage brightness and chroma. In another study on this species, Siefferman and Hill (2005a) found a positive correlation between male

mean provisioning rates (number of provisions/minute) to offspring and male mean structural-plumage brightness. Carotenoid-based plumage brightness in adult male and female northern cardinals (*Cardinalis cardinalis*) was positively correlated with the proportion of nestling feedings per hour (Linville et al. 1998). However, Smiseth et al. (2001) found that structural and melanin-based plumage brightness, chroma, and hue were not associated with paternal care in common bluethroats. Their methodology differed from the one used in my study because they calculated individual colorimetric variables instead of using principal component analysis.

The only study that examined European starling plumage spectral characteristics and their association with parental care was by Komdeur et al. (2005). Their PC1 scores were negatively correlated with male feeding rate to the offspring; however, their hackle analysis differed from the one used in my study. Komdeur et al. (2005) incorporated hackle length (a known predictor of starling age) as a variable in their PCA, and in so doing they did not look strictly at spectral characteristics. Also, they did not separate the multiple-peak hackle reflectance data into bins along the avian-visual spectrum, which I did, but instead calculated colorimetric variables (brightness, hue, and chroma).

Adult female European starlings appear to be receiving the direct benefit of having a good provider for their offspring by selecting a social male with brighter hackles over a social male with duller hackles. Hackle brightness may have evolved as an honest signal of parental quality that can be used in female

mate choice. Additionally, older males in the population have learned throughout their breeding years how to be a good parent (incubating the eggs, protecting and provisioning the young) (Westneat and Sherman 1993), and their hackles are longer than those of younger males (Komdeur et al. 2005). If male hackle brightness increases with age then brightness would directly signal paternal experience to females. Additionally, if male hackle brightness increases with age, then brightness would signal how good males are at surviving as parents since good fathers use their time to forage for their young and not themselves, and expose themselves to predators in the process. Male plumage brightness could act as an honest signal of paternal care and viability, thus being a phenotypic signal of good genes. Females would then receive the direct benefit of having well-fed young by being paired with a good social mate and the indirect benefit of within-pair young inheriting their father's parenting and viability genes. Examining the role of age in adult male and female European starling ornaments is the next logical step in research with our study population.

I found no correlation between hackle brightness or color in females and adult female provisioning effort to their offspring. Few studies have examined the relationship between female plumage spectral characteristics and parental care. However, Siefferman and Hill (2005b) found adult female eastern bluebirds with more colorful structural-blue plumage on their rumps made more provisioning trips to their nestlings than did females with less colorful rumps. My results suggest that adult female starlings in this population are not signaling their

parental quality through hackle spectral characteristics, but are signaling their phenotypic condition through hackle brightness.

### **Reproductive Success**

Male hackle spectral characteristics did not correlate with male realized reproductive success (when nestlings were 5-6 days old) or with the proportion of within-pair young produced. Although I did not examine fledging success, Komdeur et al. (2005) found male hackle spectral characteristics were not correlated with fledging success in European starlings, however they did not look at realized reproductive success. This finding contrasts that of Siefferman and Hill (2005c), who found that male eastern bluebirds with high UV-chroma fledged significantly more offspring from their nest compared with males having low UV-chroma. In addition, Bitton et al. (2007) found that in tree swallows (*Tachycineta bicolor*), both male age and plumage brightness were positively correlated with the number of sired extra-pair young that successfully fledged. Therefore, my results do not support the hypothesis that male hackle spectral characteristics are correlated with male realized reproductive success or with the proportion of within-pair young produced in this population of European starlings.

There are several potential explanations for why I did not detect a relationship between adult male hackle spectral characteristics and male realized reproductive success. My estimate of male realized reproductive success is conservative in that it only accounts for nestlings that were produced in

nestboxes and not those that were located in tree cavities or in buildings, which are difficult to access. Finally, some other ornament (e.g. bill color) may be responsible for predicting male realized reproductive success.

Male song in all oscines has evolved to attract mates. European starlings are open-ended song learners (Eens et al. 1991). Consequently, males are able to extend song learning over their lifetime, with repertoire size increasing with age (Mountjoy and Lemon 1995). Females preferred males with larger song repertoire sizes, and laid their eggs earlier in the breeding season when paired with these males as compared to females paired with males singing a shorter repertoire (Mountjoy and Lemon 1996). European starling song quality is a trait that signals both adult male viability (age) and the capacity to learn songs to attract mates. Future studies should analyze if male realized reproductive success is correlated with song characteristics (e.g. song bout length, strophe length, and repertoire size), and should examine if European starling song characteristics are correlated with hackle spectral characteristics.

Timing and food availability have been shown to influence the reproductive success in a variety of birds (e.g. Verhulst et al. 1995; DeForest and Gaston 1996; Siikamaki 1998). The removal of first clutches in great tits (*Parus major*) has been shown to lower the reproductive success in second clutches (Verhulst et al. 1995). Experimentally altered hatching dates in pied flycatchers have been shown to lower their reproductive success (fledging success); however, if they were given an extra-food supply, the treatment adults were able to successfully

fledge as many young just as the control adults (Siikamaki 1998). Future studies would benefit from manipulating clutch sizes and altering laying times of European starlings and examine how these non-genetic factors can affect reproductive success.

Komdeur et al. (2005) and Bitton et al. (2007) incorporated age with plumage characteristics as a predictor of male reproductive success. They found that older males with high-quality feather characteristics had a greater reproductive success than younger males. Females that mate with older males displaying high-quality plumage are thought to be increasing their evolutionary fitness by indirectly acquiring good viability genes for their offspring. Future studies should examine if male and female starling age is correlated with their hackle spectral quality and whether or not their realized reproductive success increases with age.

Female hackle color was positively correlated with female realized reproductive success. Females with purpler hackles produced more young in the study population than did females with greener hackles. This result is comparable to that of Komdeur et al. (2005) who found that brighter and purpler females fledged more offspring than did duller and greener ones; however, their study did not look at female realized reproductive success. Komdeur et al.'s (2005) study was the first to discover that purple hackles of adult female European starlings correlated positively with fledgling output. My study is the first to discover that females with purple hackles had the highest realized reproductive success (when

nestlings were 5-6 days of age) in comparison to females with green hackles. Siefferman and Hill (2005b) found that adult female eastern bluebirds with high-quality structural- and melanin-based plumage (e.g. high brightness, chroma, and hue) fledged more offspring than females with low-quality plumage; however, my study did not look at fledging success.

### **Sex Allocation**

The brightest males sired a higher proportion of male genetic offspring in comparison to duller males who produced proportionally more females. Color or PC3 scores in both males and females did not play a role in determining nestling sex ratios. Sheldon et al. (1999) found that male blue tits with high UV-chroma and hue on their crown feathers sired significantly more male than female offspring in comparison to males that had low UV-chroma and hue. Dreiss et al. (2005) found that song was a predictor of primary sex ratios in blue tits. Male blue tits with larger strophes and longer bouts (time singing between breaks) produced proportionally more sons than daughters. Likewise, Potvini and MacDougall-Shackleton (2010) found that male song complexity in song sparrows (*Melospiza melodia melodia*) was positively correlated with a male-biased sex ratio close to fledging (secondary sex ratio).

Sex allocation theory in birds suggests that heritable phenotypic traits, such as plumage, can act as a cue for females to produce more male or female young pre- or post-egg development (e.g. Komdeur and Pen 2002). My results

suggest that female European starlings are adjusting the ratio of eggs ovulated in accordance to the brightness of male hackles. The brightness of male hackles may act as a signal of genetic quality. Females would benefit by producing more male offspring to inherit their father's plumage characteristics. Ellegren et al. (1996) found that the proportion of male nestling pied flycatchers at hatch produced by a female was positively correlated with the size of her mate's forehead patch area (a sexually selected trait in male pied flycatchers). They hypothesized that the male forehead patch size may be a Fisherian trait (i.e. "sexy" trait; see Introduction), whereby the trait's sexual attractiveness continues to amplify based on the preference that females have for it. They were unable to confirm if the trait indirectly reflected a male's viability, and they did not analyze the paternity of the offspring. Regardless, a female would receive genetic benefits and increase her own fitness by producing more sons to inherit their father's attractive traits, rather than producing more daughters. My study is comparable to that of Ellegren et al.'s (1996) because female European starlings may be using hackle brightness as a criterion for mate attractiveness. My study did not investigate the viability of nestlings or the age of adults, so I am unable to infer if hackle brightness is indirectly expressed by good genes. However, I can infer that hackle brightness in males is a signal of their overall genetic quality (e.g. compatible or mate-attracting genes). A male-biased sex ratio may also be due to males typically having a greater variance in reproductive success than females (e.g. Ligon and Hill 2010), so it would be beneficial for mothers to produce more

sons when mothers are in good condition and/or during good environmental conditions. Future studies should look at assortative mating to see if older males with high-quality hackles are paired with older females with high-quality hackles, and determine if this pairing correlates with a male-biased sex ratio.

Female hackle characteristics did not correlate with the proportion of male offspring that females produced. These results suggest that female phenotypic characteristics do not influence nestling sex ratios. However, it would be important to examine whether assortative mating exists and if it correlates with hackle spectral characteristics.

## **Conclusions and Future Direction**

My data support that hackle spectral characteristics indirectly signal an individual's body-condition through hackle color (males) and brightness (females). They also suggests that males with brighter hackles are better parents than males with duller hackles, and that females with purple hackles genetically produce more offspring than do females with greener hackles. Furthermore, it suggests that brighter males tend to genetically produce proportionally more male offspring than duller males. The results also support that hackle spectral characteristics are an important cue used in mate choice for European starlings and that they indirectly signal an individual's genetic quality; however, I cannot infer that hackle spectral characteristics indirectly reflect good genes as I did not analyze adult age or nestling recruitment. Future studies need to investigate age as a potential contributor to mate choice in European starlings because age appears to play a pivotal role in plumage quality, parental quality, and reproductive success in other passerines (e.g. Bitton et al. 2007; Probst et al. 2007; Bitton and Dawson 2008; Budden and Dickinson 2009; Edler and Friedl 2012). In addition, nestling recruitment would be difficult to determine because the study population with which I worked has a low level of natal philopatry (unpublished data).

European starling song should also be examined as a predictor of male parental quality, realized reproductive success, and its effect on nestling sex

ratios. I recommend that future studies investigate if there is a correlation between high-quality hackle spectral characteristics and song. In so doing, hackle spectral characteristics may provide further evidence that male phenotypic characteristics used in mate choice reflect an individual's genetic quality. I also recommend that future studies test the Hamilton-Zuk hypothesis (parasite-mediated sexual selection) and examine if hackle spectral characteristics in adult European starlings correlate with their parasite load. By looking at parasites and plumage, one could determine if hackle characteristics signal an individual's immunocompetence, which is indirectly expressed by good genes.

In conclusion, my study provides supportive data that hackle spectral characteristics in European starlings are used in mate choice. It also further strengthens the understanding that plumage characteristics have evolved under sexual selection in birds (Darwin 1871), and that there are benefits for those who mate with males or females displaying plumage of high-spectral quality.

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