PLASMA LEPTIN IN RELATION TO BREEDING STAGE, SEASON, ESTROGEN AND PHOTOPERIOD IN FEMALE EUROPEAN STARLINGS (*STURNUS VULGARIS*)

by

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ABSTRACT

Leptin, a protein hormone secreted by adipocytes, has wide-ranging physiological functions, including regulation of feeding behavior and body weight, and effects on reproduction and immune function. Our first study demonstrated seasonal and breeding-stage patterns of variation in plasma leptin-like immunoreactivity in free-living female starlings (*Sturnus vulgaris*), which did not parallel changes in body mass and composition. Plasma leptin-like immunoreactivity was elevated from egg-laying through clutch completion, decreased during incubation and chick-rearing, and was elevated in non-breeders in November. Next, we manipulated wild-caught females to determine whether elevated plasma leptin-like immunoreactivity is associated with a) seasonal, photoperiodic (long day length-dependent) reproductive development, or b) elevated estrogen required for egg production. Plasma leptin-like immunoreactivity was unaffected by photoperiod or estradiol treatment; however, because estradiol treatment on 18L:6D only incompletely stimulated the reproductive axis, we cannot unequivocally exclude a link between elevated plasma estradiol and plasma leptin-like immunoreactivity during egg-production in free-living birds.

Keywords: leptin, photoperiod, estradiol, reproductive axis, European starling (*Sturnus vulgaris*)

Subject Terms: leptin, avian, reproduction, European starling (*Sturnus vulgaris*)

Dedicated to Jon, Bailey and my entire family

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Chapter 1 :
INTRODUCTION

1.1 Overview of Leptin Research in Mammals and Birds

Leptin, a hormone secreted by fat cells – which is also expressed, to a lesser extent, in gastric epithelium and placenta - has been extensively studied in mammals since the ob gene encoding leptin was first cloned and sequenced (Zhang et al. 1994, Masuzaki et al. 1997, Bado et al. 1998, reviewed in Friedman and Halaas 1998). Leptin is best known as an adiposity signal - wherein the expression and secretion of leptin by adipocytes, as well as plasma leptin concentrations, positively correlate to the quantity of adipose tissue - and for its role in feeding regulation in mammals (Halaas et al. 1995, Considine et al. 1996). Mice with an ob gene mutation that do not express the gene are severely obese, but administration of leptin into these individuals results in decreased food consumption and increased energy expenditure (Zhang et al. 1994, Halaas et al. 1995). However, leptin's actions in mammals also include reproduction (Ahima et al. 1996, Henry et al. 2001, Steiner et al. 2003), and immune function (Lord et al. 1998), and leptin may also exhibit circadian (Klingenspor et al. 2000, Marie et al. 2001) and seasonal patterns (Hissa et al. 1998, Nieminen et al. 2001). Leptin has also been studied in birds, however; because the gene sequence for purported avian leptin has not been found in the chicken genome, avian leptin research is highly controversial (Friedman-Einat et al. 1999, Sharp et al. 2008). In agreement with the verisimilitude of avian leptin's existence, injections of mammalian and purported avian leptin into birds produce

similar effects in birds as have been documented in mammals (see below). Purported avian leptin studies have demonstrated this hormone's effects pertaining to feeding regulation and adiposity signalling (Denbow et al. 2000, Lohmus et al. 2003, Kuo et al. 2005, Lohmus et al. 2006), as well to as immune function (Lohmus et al. 2004, Alonso-Alvarez et al. 2007) and reproduction (Paczoska-Eliasiewicz et al. 2003, Paczoska-Eliasiewicz et al. 2006, Sirotkin and Grossmann 2007, Sirotkin et al. 2007). Furthermore, whether this hormone is actually avian leptin or some other hormone, it has wide-ranging effects that have not been fully explored.

1.2 Physiological Effects of Leptin

Leptin is involved in fat regulation, and variations in plasma leptin can alter feeding behavior; in mammals, the amount of leptin circulating in an individual's plasma positively reflects the quantity of adipose tissue, with high circulating levels signalling a long term decrease in feeding behavior (reviewed in Friedman and Halaas 1998). For example, wild-type mice injected with leptin had decreased feeding behavior, body mass, and body fat (Halaas et al. 1995). Comparable results have been shown in birds; Asian Blue Quail (*Coturnix chinensis*) that received purported recombinant chicken leptin through osmotic mini-pumps for two weeks exhibited decreased feeding behavior and body mass (Lohmus et al. 2006). Furthermore, immunization against purported recombinant chicken leptin results in increased food intake, body mass, and body fat in chickens (Shi et al. 2006). Thus, there is substantial evidence to support the idea that purported avian leptin moderates feeding behavior and reflects adiposity levels in birds.

The role of leptin as an adiposity signal is also linked to its roles in immune function (reviewed in La Cava and Matarese 2004). Individuals without fat reserves are

unable to produce a strong immune response; therefore, starved individuals suffer from immunosuppression (Chandra 1991). Additionally, inflammatory cytokines have been shown to increase serum leptin levels in mice (Sarraf et al. 1997). The connection between fat stores, immune function and leptin was successfully demonstrated when starved mice administered with leptin were rescued from immunosuppression by leptin's induction of T-lymphocyte proliferation (Lord et al. 1998). To my knowledge, there has been very little research on leptin's role in immune function among birds; however, Lohmus et al. (2004) demonstrated that administration of purported chicken recombinant leptin in Asian blue quail facilitated an enhanced immune response, such that leptin-treated individuals had increased T-lymphocyte proliferation following a PHA wing-web test. Additionally, recombinant murine leptin injections have been shown to rescue Zebra Finches (*Taeniopygia guttata*) from immunosuppression induced by testosterone implants (Alonso-Alvarez et al. 2007). Thus, it appears as if leptin is related to immune function through adiposity in a similar manner in birds as in mammals.

There is also research to suggest that adiposity provides a connection between leptin and reproduction; leptin has been suggested as the link between fat reserves and both the maintenance of reproductive function as well as the onset of puberty in mammals. Specifically, it has been theorized that once a critical percent body fat has been reached, the individual undergoes the onset of puberty, and circulating levels of leptin - corresponding to percent body fat - may either permit or in part trigger reproductive development (reviewed in Kiess et al. 1998, Chehab 2000, Budak et al. 2006). In human females with anorexia nervosa (AN), leptin levels are low (Grinspoon et al. 1996), and while individuals with AN often fail to menstruate, in recovering AN

females, recovered leptin concentrations may be a necessary component for resumed menstruation (Audi et al. 1998). Additionally, administration of leptin in starved female mice prevented starvation-induced delayed ovulation (Ahima et al. 1996). Similarly, in chickens, injections of purported recombinant chicken leptin caused advanced puberty, indicated by first oviposition (Paczoska-Eliasiewicz et al. 2006). Finally, injections of purported chicken recombinant leptin into hens attenuated adverse reproductive effects due to fasting, including: cessation of egg-laying, decreases in LH, progesterone, and estradiol, follicular regression and apoptosis (Paczoska-Eliasiewicz et al. 2003). In summary, both birds and mammals seem to exhibit a similar link between adiposity, reproduction, and leptin.

Furthermore, leptin appears to be involved in other aspects of avian reproduction in addition to its link with adiposity (see below). Firstly, Paczoska-Eliasiewicz et al., (2003) have identified leptin receptors in hen ovaries. Also among hens, the secretion of several reproductive steroids, cell apoptosis and proliferation were altered by injections leptin or culturing ovarian cell walls or granulosa cells with human recombinant leptin (Sirotkin and Grossmann 2007, Sirotkin et al. 2007). Similarly, advanced pre-pubertal increases in plasma LH, progesterone and estradiol were demonstrated in purported recombinant chicken leptin-administered pullets fed ad libitum (Paczoska-Eliasiewicz et al. 2006). Therefore, there is some evidence to support leptin's involvement with secretion of reproductive steroids in birds. Additionally, leptin may be involved in some aspects of frequency of reproductive effort, as immunisation against purported recombinant chicken leptin resulted in decreased laying rates among hens (Shi et al. 2006).

Leptin also exhibits circadian and seasonal patterns of change among some mammals, and photoperiodic responses may be linked with reproduction. Seasonal patterns (Hissa et al. 1998, Concannon et al. 2001, Nieminen et al. 2001, Nieminen et al. 2002, Mustonen et al. 2005) in serum leptin have been documented in several mammalian species, but the mechanisms regulating seasonal patterns in leptin have not been elucidated. Additionally, some studies have examined serum leptin levels on different day lengths in Siberian or Djungarian hamsters (*Phodopus sungorus*) or Soay rams (*Ovis aries*) (Atcha et al. 2000, Drazen et al. 2000, Horton et al. 2000, Klingenspor et al. 2000, Marie et al. 2001). In particular, one study determined that gonadally regressed male Siberian hamsters housed on a short day length (8L:16D) had low leptin levels, in contrast to males on both short and long day lengths (16L:8D) that had functional gonads and had high leptin levels (Drazen et al. 2000). This study exemplifies that plasma leptin levels can be linked to photostimulatory gonadal responsiveness. However, daily and seasonal fluctuations in plasma leptin-like immunoreactivity in addition to photoperiod-induced reproductive responsiveness remain unexplored in birds.

1.3 Leptin in Birds – The Unresolved Controversy

Although there are many studies claiming to measure avian leptin as well as demonstrating the effects of leptin administration in birds (see above), avian leptin research is highly controversial. The controversy stems from the debate over the cDNA sequence of purported chicken leptin in GenBank (chicken leptin gene GenBank Accession Number: AF012727; Taouis et al., 2008). Some researchers believe that the high degree of sequence similarity of the purported chicken leptin gene to rodent leptin gene sequences is so evolutionarily improbable that the sequence is erroneous (see

below). However, there is also ample evidence to suggest that leptin or a leptin-like molecule does exist in birds, and that regardless of whether the current sequence in GenBank is correct, the high similarity in mammalian and avian sequences enables both mammalian and putative avian leptins to induce physiological responses in birds (see below).

Taouis and colleagues (1998) were the first to clone and sequence the purported chicken leptin gene, and furthermore, showed that in chickens this gene is expressed in liver as well as adipose tissue (unlike mammals). Chickens may have putative leptin expression in the liver because the liver is the major site of lipogenesis (Taouis et al. 1998, Ashwell et al. 1999b, Dridi et al. 2005, Kochan et al. 2006), and specifically vitellogenesis, during egg production. Additionally, findings from various hormonal manipulations designed to alter putative leptin expression in hepatic and adipose tissue indicate that different mechanisms could be responsible for regulating expression in these two locations; conversely, because none of the hormone treatments increased purported leptin expression in adipose tissue, expression in this tissue could constantly be at its peak (Ashwell et al. 1999a).

Primers from the mouse ob gene published by Zhang et al. (1994) were used to clone the gene from RNA in chicken liver and adipose tissue (muscle RNA failed to amplify), and the reported coding sequences for these two types of tissue are the same (Taouis et al. 1998). Ashwell et al. (1999a) also reported putative leptin expression in chicken liver and adipose tissue, and they also demonstrated expression in chicken yolk sack and embryonic liver. Ashwell and colleagues (1999a) cloned purported chicken leptin in chicken liver and adipose tissue using primers that they choose from sequence

sections retaining high similarity across many mammal species as well as the same primers used by Taouis et al. (1998) for amplification. The sequence published by Ashwell et al. (1999a) has only a single nucleotide difference from the sequence published by Taouis et al. (1998), and both nucleotide sequences code for an identical amino acid sequence. Ashwell and colleagues (1999a) suggest that the nucleotide sequence difference could be due to the fact that they used a different strain of chicken than Taouis and colleagues (1998). Taouis and colleagues (1998) report that the degree of similarity between the protein sequence (amino acid sequence without any gaps) of the purported chicken leptin gene and mouse leptin gene is 97%, and 83% to the human leptin gene, although they acknowledge that there is a high degree of evolutionary distance between birds and mammals.

Conversely, Friedman-Einet et al. (1999) attempted to amplify the putative avian leptin gene from chicken RNA and genomic DNA using 14 different mouse primers, including the primers used by Taouis et al. (2008), but could not make avian PCR products that were similar to the murine sequence, and they assert that hepatic and adipose tissue of chickens, turkeys, geese and Japanese quail do not express mRNA for a gene sequence closely resembling this murine sequence. Friedman-Einet and colleagues (1999) also dispute the appropriateness of the mouse leptin primers employed by Taouis et al. (1998): (a) because the forward primer is not identical to the murine sequence, and (b) because the primers are from an un-translated portion of the cDNA, and these regions are usually inappropriate for amplification in species that are evolutionary distant because these regions are less evolutionary conserved. Furthermore, the authors demonstrated that mouse leptin probes did not hybridize to chicken or goose RNA (unsuccessful

northern hybridization) or chicken DNA (unsuccessful southern hybridization under conditions of high stringency washing) (Friedman-Einat et al. 1999). However, southern hybridization was successful when low stringency washing conditions were implemented, which could either be attributed to the presence of a supposed chicken leptin gene or to cross-hybridization to other portions of the DNA; yet, under medium stringency conditions, only the mouse and sheep signals remained (Friedman-Einat et al. 1999). Thus, the authors assert that the purported chicken leptin sequence must be less than 83% identical (sheep and mouse are 83% identical) to the murine sequence, unlike the sequences published by previous researchers (Friedman-Einat et al. 1999).

Subsequently, Doyon and colleagues (2001) performed phylogenetic analyses on portions of the leptin nucleotide sequences first from six mammals, and then they added the sequences for purported chicken and turkey leptins; while the phylogenetic grouping of the mammalian leptin sequences (when only mammalian sequences were included in analysis) does concur with the established evolutionary relationships among mammals, when purported chicken and turkey leptin are added to the tree, chicken and turkey leptin were placed with rodent leptins. Namely, the level of similarity for the putative chicken leptin sequence with mouse leptin is higher than the similarity between leptin sequences among mammals, although it is widely accepted that mice are more closely evolutionarily related to other mammals than they are to birds (Doyon et al. 2001). The authors propose that convergent or parallel evolution of the rodent and putative avian leptin sequences could account for the exceptionally high resemblance between these sequences (Doyon et al. 2001). However, while the authors suggest a common ancestral source for putative

avian leptons and rodent leptins, they do not propose any possible selective forces for this proposed convergence (Doyon et al. 2001).

Purported leptin homologs have been found in other non-mammalian vertebrates, for example, in the African clawed frog (Crespi and Denver 2006). The amino acid sequence similarity of putative frog leptin to mammalian leptins is only ~35% even though the proteins' structures are highly similar and the frog protein also suppresses appetite (during part of the frog's life cycle), like mammalian leptins (Crespi and Denver 2006). Similarly, the Tiger salamander (*Ambystoma tigrinum*) has a leptin-like gene that is 29% identical to the amino acid sequence of mammalian leptins, but the protein structures are more similar (Boswell et al. 2006). Additionally, the authors assert that unlike putative chicken leptin, salamander leptin has a non-synonymous substitution rate that concurs with well-established evolutionary divergence among vertebrates (Boswell et al. 2006). Furthermore, Boswell and colleagues (2006) ask if the leptin gene is merely undiscovered in birds or if it is not present in birds and has perhaps been lost. It is possible that putative avian leptin is not orthologous (homologous gene sequences in diverged species) to mammalian leptin, meaning, they are not homologous sequences that were separated by speciation. Perhaps, although other vertebrates have leptin homologs, birds could have lost this gene, and they instead possess an analogous hormone. Thus, purported avian leptin could simply be a leptin-like hormone that performs similar functions to leptin with receptors similar to avian leptin receptors, so injections of mammalian leptin into birds yield leptin-like effects.

Sharp et al. (2008) presented a distribution analysis of synonymous substitutions for 20 homologous chicken and leptin genes (from Dunn et al., 2001) to argue that the

remarkable resemblance between the mouse leptin and putative chicken leptin cDNA sequences is improbable in the evolutionary sense. A frequency distribution of these synonymous substitutions revealed that the odds that the putative sequence of the chicken leptin gene is contained in the curve are less than 1 in 1 million; therefore, the authors attest that it is highly likely the purported cDNA sequence for chicken leptin is erroneous (Sharp et al. 2008). In addition, nucleotide substitution data for leptin genes across taxa were compiled to create a phylogenetic tree (from Dunn et al., 2001) which illustrated higher similarity between the chicken and the mouse sequences than between the mouse sequence and other mammal and marsupial sequences (Sharp et al. 2008).

The controversy surrounding avian leptin revolves solely around the current gene sequence in GenBank. It is possible the sequence is erroneous (see arguments above regarding similarity to mouse leptin), although two independent labs have published the same amino acid sequence for putative chicken leptin (Taouis et al. 1998, Ashwell et al. 1999a). Doyon and colleagues (2001) assert that it is improbable that two labs that cloned and sequenced putative chicken leptin had the same cloning error due to mouse contamination - as proposed by Dunn et al. (2001) - and furthermore suggest that an even greater sequence resemblance would have resulted from a theoretical contamination. Thus, Doyon et al. (2001) contest that the current putative chicken leptin sequence is likely correct. One possible explanation for the present sequence's absence in the genome is that the leptin receptor is so highly conserved across taxa, that injections of purported avian leptin into birds are recognized as leptin and induce relevant biological responses even if the current sequence has some discrepancies from the sequence encoded in the chicken genome (which has not yet been identified) (McMurtry, pers.

comm.). This possible explanation is also in agreement with studies that have demonstrated that injections of mammalian leptin into birds produce responses similar to those found in mammals (see below).

Despite the controversy surrounding the purported sequence of chicken leptin, there is evidence to suggest that leptin, or a similar hormone, is endogenous to birds. Firstly, as asserted by Doyon et al. (2001), a chicken leptin receptor gene (known as CLEPR or chLEPR or cOB-R) has been cloned (Horev et al. 2000, Ohkubo et al. 2000). Yet, in contrast to the amino acid sequence for putative chicken leptin, the chicken leptin receptor gene has only 49% amino acid similarity to the mouse homolog and has on average, 50% amino acid similarity to other mammalian leptin receptors; whereas, the mammalian leptin receptor genes share a higher degree of resemblance with each other (Horev et al. 2000). Therefore, Horev and colleagues (2000) attest that the reported chicken leptin gene sequence, with a 97% identical amino acid sequence to mouse leptin, is incorrect. However, the structure of the chicken leptin receptor gene, specifically the exons, greatly resembles mammalian leptin receptor genes (Horev et al. 2000). Additional support for the existence of avian leptin was provided by Ohkubo and colleagues (2000), who demonstrated ovarian and hepatic expression of chicken leptin receptor mRNA as well as expression in the kidneys, intestines, and brains of hens. Furthermore, Paczoska-Eliasiewicz et al. (2003) found expression of chicken leptin receptor mRNA in the ovary, hypothalamus, and pituitary gland of hens, and described the quantity of expression in different ovarian follicles. Also, the chicken leptin receptor is capable of binding mammalian leptin as well as mediating the leptin signal *in vitro* (Adachi et al. 2008, reviewed in Ohkubo and Adachi 2008). Additional evidence for the

existence of leptin in birds is provided by studies demonstrating that mammalian and putative avian leptin have been shown to elicit physiological responses in birds. For example, as asserted in Scanes (2008), human recombinant leptin decreased feeding in chickens (Denbow et al. 2000, Kuo et al. 2005); additionally, purported chicken recombinant leptin decreased feeding behavior in Asian Blue quail (Lohmus et al. 2006) and Great tits (*Parus major*) (Lohmus et al. 2003).

While there is ample evidence reporting similar physiological responses to leptin in birds as in mammals, as well as research purporting measurement of variation in plasma leptin in birds and the existence of a leptin receptor(s), as reviewed by Scanes (2008), the substantial controversy surrounding avian leptin has spurred Sharp and Colleagues (2008) to request that the journal General and Comparative Endocrinology not except papers on purported avian leptin until avian leptin's existence (both the gene and the encoded protein) is proven. While it is essential that researchers resolve the controversy surrounding the existence of avian leptin by confirming the current cDNA sequence or determining the correct cDNA sequence, it is absurd to request that no research examining supposed circulating leptin levels or administering exogenous leptin in birds be accepted until the sequence is resolved, because this research has illuminated interesting patterns in a hormone (regardless of whether it is avian leptin) that must be more fully explored, even if the sequence coding this hormone is unknown. The Editor in Chief of the journal Poultry Science, Dr. C.G. Scanes, not only does not support Sharp and colleagues' request to reject avian leptin articles of this nature, he instead attests the high likelihood of the existence of avian leptin (he evidences leptin receptor(s) in birds, similar effects of exogenous leptin in birds and mammals, reports of leptin expression in

birds, etc.), and asserts that it is more probable that either the current sequence is correct or the actual sequence is unknown (Scanes 2008). He suggests researchers acknowledge the controversy surrounding avian leptin and tentatively use terms such as "leptin-like" or "leptin immunoreactivity" to describe avian leptin (Scanes 2008). Therefore, in this thesis, I report leptin-like variation in plasma concentrations among European starlings (*Sturnus vulgaris*), which I will refer to as plasma leptin-like immunoreactivity.

1.4 Study Species - *Sturnus vulgaris*

The following description of European starlings is from a review in The Birds of North America (Cabe 1993). European starlings are an extremely pervasive non-native species that is distributed throughout North America; however, as they are less likely to live in mountains and forests, they are less evenly distributed in the west. Additionally, they are wide-spread in many places around the world, and they have successfully colonized many places where they have been introduced. Starlings are cavity nesters, which out-compete native bird populations, and they nest in natural cavities or in man-made holes, such as in buildings, or in nest boxes. Starling flocks forage in agricultural or in urban areas on short grass fields or lawns and consume many types of food including fruits, berries, grains, seeds, and invertebrates (though invertebrates are preferred). Both males and females fight for food, and males also fight for breeding sights. Males are the more predominant sex among adult populations (2:1) and sing to attract females to their nest site. Starlings have a wide repertoire of vocalizations and can imitate other avian and even non-avian species and inanimate sounds. Monogamy is the most prevalent mating system; however, many populations demonstrate polygny. Males are vigilant of their mates to prevent extra-pair copulations. Males also feed and brood

the nestlings of their primary mates more than nestlings of their secondary mates (Cabe 1993).

Populations lay their first clutches synchronously. Some individuals will lay a second clutch soon after the first clutch fledges (avg. 6-10 days post fledging). Often, there are also intermediate nests - between the first and second clutch - that are destroyed. North America clutch size means range from 4.28-5.15. Eggs are usually laid in the morning. Females begin to incubate when they lay their final egg (though males do some incubation as well), and the duration of incubation is approximately 12 days. Nestlings are primarily fed in the morning and late afternoon by both parents. Nestlings are also brooded by both parents (though primarily by the female) for approximately one week during the day and night, and nestlings can thermoregulate by 13 days after hatching. Nestlings fledge between 21-23 days after they hatch, and though they can fly, they still receive some of their food from their parents near the nest. However, after 10-12 days they forage for themselves (Cabe 1993).

Both male and female starlings have extremely high breeding site fidelity (Cabe 1993), which makes them ideal for field research because a researcher may be able to collect breeding season data several times for the same bird over a multiyear study at a field site. Additionally, because starlings breed at sites in large flocks, a field site can provide a large sample size. Furthermore, starlings readily nest in boxes, which allows reasonably simple capture in the field. Because females brood their clutches at night, they are particularly straight-forward to capture, although both males and females can be captured when they feed their young. Starlings also adapt quickly to captivity without

apparent body mass drops (Chapter 3), so starlings are a low maintenance species on which to perform directed captivity studies.

Starling beak coloration is altered by changes in hormone concentrations associated with the breeding season (yellow in breeding season), and hormone concentrations are driven by photoperiod; additionally, they are a model species for studies on photoperiodic effects on hormone levels (Cabe 1993). Because photoperiod and reproductive hormone changes have been explored in this species, they are an appropriate model for our examination of variation in leptin-like immunoreactivity during the breeding season as well as our study on possible photoperiodic and estrogenic effects on leptin-like immunoreactivity in birds. While leptin's functions appear to be similar in birds and mammals, because putative leptin expression has been found in the liver as well as in adipose tissue in birds, unlike mammals (Taouis et al. 1998; see above), and because different mechanisms could be responsible for regulating expression in hepatic and adipose tissue (Ashwell et al. 1999a); it is important to measure putative leptin in birds as well as mammals. Thus, putative avian leptin research is important because not all leptin findings in mammals can necessarily be extended to birds, and putative avian leptin appears to have many important roles in birds. It is crucial to explore the roles of all hormones in the endocrine system to understand the holistic physiology and ecology of a species, thus research on putative avian leptin is necessary.

1.5 Thesis Objectives

My research aims were to examine patterns of change in avian plasma leptin-like immunoreactivity (measured by Radioimmunoassay) during the reproductive and non-reproductive seasons among free-living female European starlings (Chapter 2), with an

additional directed experiment on wild-caught individuals investigating possible stimulatory effects of estrogens and photoperiod on plasma leptin-like immunoreactivity (Chapter 3). Because all published research on purported avian leptin (to date) investigates patterns exclusively among captive birds; the research described in this thesis is distinctive because it explores variation in plasma leptin-like immunoreactivity among both captive and free-living birds. The objective of the captive study is to explore possible mechanisms causing variation in plasma leptin-like immunoreactivity found among free-living individuals, and the findings from this captive experiment will be placed in an ecological context.

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Chapter 2 :
SEASONAL AND REPRODUCTIVE STAGE VARIATION IN PLASMA LEPTIN-LIKE IMMUNOREACTIVITY IN FREE-LIVING EUROPEAN STARLINGS (*STURNUS VULGARIS*)

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2.1 Abstract

Leptin, a protein hormone secreted by fat cells, is best known for its role as an adiposity signal; however, leptin has diverse physiological roles ranging from regulation of feeding behavior and body weight, to effects on reproduction and immune function. Although leptin has been extensively studied in mammals, purported avian leptin remains highly controversial, and studies have focused on captive or domesticated species, namely chicken and quail. In this study, we describe changes in plasma leptin-like immunoreactivity during the reproductive and non-reproductive seasons in free-living female European starlings (*Sturnus vulgaris*). Plasma leptin-like immunoreactivity was high during egg-laying (27.8 ± 2.4 ng/mL) and clutch completion (23.8 ± 1.6 ng/mL),

decreased during incubation (13.0 ± 1.6 ng/mL) and chick-rearing (12.0 ± 1.3 ng/mL), and were again elevated in non-breeders in November (23.7 ± 1.1 ng/mL). Although there was marked and consistent variation in total body mass and body composition with breeding stage and season in this population, patterns of variation in plasma leptin-like immunoreactivity did not parallel changes in body mass or body composition. In conclusion, our findings demonstrate both reproductive stage and seasonal variation in plasma leptin-like immunoreactivity, which are disparate to patterns of change for body mass and body composition, suggesting that the relationship between plasma leptin-like immunoreactivity and body mass reported for captive birds and mammals does not hold for free-living birds such as the European starling. Furthermore, in the context of research linking putative avian leptin with reproduction, we discuss whether elevated estradiol levels during egg-formation could be responsible for the high plasma leptin-like immunoreactivity we found during egg-laying.

2.2 Introduction

Leptin, a protein hormone secreted by fat cells (adipose tissue), was discovered and identified as the product of the obese (ob) gene that was sequenced and cloned in mice and humans less than 15 years ago (Zhang et al. 1994). Since then, a very large amount of research, mainly in mammals, has demonstrated that leptin has extremely diverse physiological roles ranging from regulation of feeding behavior and body weight, to effects on reproductive function and the immune system (Halaas et al. 1995, Ahima et al. 1996, Considine et al. 1996, Lord et al. 1998, Steiner et al. 2003). Few mammalian studies—except for those involving humans— have examined leptin among free-living individuals (but see Kunz et al. 1999), but there is some evidence to suggest that plasma

leptin can vary "seasonally" in some captive mammals (Hissa et al. 1998, Concannon et al. 2001, Nieminen et al. 2001, Nieminen et al. 2002, Mustonen et al. 2005). For example, plasma leptin levels in the Blue fox (*Alopex lagopus*) are relatively low during the summer, increase during the fall when individuals accumulate body fat, and then decrease during the winter and spring when the animals reduce their body fat (Nieminen et al. 2001, Mustonen et al. 2005). In contrast, in woodchucks (*Marmota monax*), plasma leptin is elevated in late spring and peaks in the summer before decreasing through the winter (Concannon et al. 2001).

Far fewer studies have investigated variation in, and the physiological roles of, leptin in birds or other non-mammalian vertebrates, although in birds – unlike mammals – the liver is the major site of lipogenesis (Taouis et al. 1998, Ashwell et al. 1999b, Dridi et al. 2005, Kochan et al. 2006). In part, the lack of research in birds may be due to on-going controversy on the occurrence of leptin in birds and, specifically, the putative leptin gene sequence which has been deposited in GenBank but which has not been found in the chicken genome to date (Friedman-Einat et al. 1999, Sharp et al. 2008). Nevertheless, several studies in birds have used either mammalian leptin or putative recombinant chicken leptin in manipulation studies, or have measured variation in purported plasma leptin by heterologous leptin radioimmunoassays (RIAs; based on recombinant leptin protein derived from the reported gene sequence). These studies strongly suggest that a leptin-like hormone does exist in birds with wide ranging physiological effects similar to those reported in mammals including involvement in feeding behavior, body mass, reproduction and immune function (Denbow et al. 2000, Lohmus et al. 2003, Lohmus et al. 2004, Kuo et al. 2005, Lohmus et al. 2006, Sirotkin

and Grossmann 2007, Sirotkin et al. 2007). For example, Lohmus et al. (2006) demonstrated that exogenous putative recombinant chicken leptin administration through osmotic mini-pumps decreases feeding behavior and body mass in Asian blue quail (*Coturnix chinensis*). In chickens, both injections of leptin and culturing of ovarian cell walls or granulosa cells with human recombinant leptin changes cell proliferation and apoptosis and affects secretion of several reproductive steroid hormones (Sirotkin and Grossmann 2007, Sirotkin et al. 2007). Purported recombinant chicken leptin has also been shown to increase the immune response in Asian blue quail (Lohmus et al. 2004) and recombinant murine leptin has been shown to 'rescue' zebra finches (*Taeniopygia guttata*) from immunosuppressive effects of testosterone (Alonso-Alvarez et al. 2007).

To our knowledge, all published avian research on purported leptin to date has involved captive birds, excepting one study that examined fat content and leptin-like immunoreactivity levels in a deceased free-living migratory dunlin (*Calidris alpina*) (Kochan et al. 2006), and most avian leptin research has involved domesticated species, such as chicken and quail. Therefore, nothing is known about leptin in free-living birds, and in particular it is not known whether plasma leptin-like immunoreactivity varies seasonally, as has been suggested in captive mammalian leptin studies. Given the marked seasonal and reproductive cycles that free-living birds exhibit, and the stochastic nature of conditions in nature relative to tightly controlled conditions in captive studies, field studies, in conjunction with captive studies, are crucial in avian leptin-like immunoreactivity research. Moreover, field studies may be especially valuable to challenge what we have learned from captive studies by placing our prior knowledge into the context of the natural world to determine whether these findings are relevant for

individuals living un-manipulated by environment or administration of exogenous substances. These studies may inform the debate, and perhaps help resolve the controversy, about the role of leptin in non-mammalian taxa (Sharp et al. 2008).

In this paper we describe changes in plasma leptin-like immunoreactivity during the reproductive and non-reproductive seasons in free-living female European starlings (*Sturnus vulgaris*), using a radioimmunoassay developed for purported recombinant chicken leptin (see Methods). Specifically, we first describe variation in total body mass, fat mass and non-reproductive mass in female starlings at different stages of breeding (egg-laying, clutch completion, incubation, and chick rearing) and in non-breeders in November. We then describe significant seasonal and breeding-stage specific variation in plasma leptin-like immunoreactivity, and compare this to the mass data to test the hypothesis that these plasma leptin-like immunoreactivity changes are driven by seasonal variation in body mass or provisioning activity (higher during chick-rearing), given the central role of leptin in the regulation of body weight and food intake. Our results suggest that the relationship between plasma leptin-like immunoreactivity and body mass reported for captive birds and mammals (Halaas et al. 1995, Considine et al. 1996, Lohmus et al. 2006, Shi et al. 2006) does not hold for free-living birds such as the European starling, but that seasonal variation in plasma leptin-like immunoreactivity is related to reproductive stage and seasonality *per se*.

2.3 Methods

2.3.1 Field Site and Study Overview

We opportunistically sampled female European starlings at Davistead Dairy Farm in Langley, British Columbia (49°10'N, 122°50'W) during the breeding season (April-

June 2007), and we sampled non-breeders in Fall (November 10, 2007). At the site, there are 190 nest boxes attached to wooden stakes or the sides of buildings, between 5-15 feet off the ground. During the breeding season, all nest boxes were monitored daily, between April 1-July 4, 2007 to count eggs, number eggs in laying order, and follow chick development. All field work was conducted following Canadian Council of Animal Care (CCAC) guidelines in accordance with Simon Fraser University Animal Care Committee (UACC) permit number 829B-96. At capture, all birds were sexed, measured (beak, wing and tarsus), weighed (± 0.1g), banded with metal and color bands (Environment Canada permit 10646), and a blood sample was taken. All blood samples were collected from the right or left brachial vein and centrifuged < 5 hours after collection, and plasma was frozen at -20 C until assay analysis.

2.3.2 Sample collection

During the breeding season, females were captured in their nest boxes for blood sampling by plugging the nest hole with a sponge attached to a long metal pole and sampled within 60 min of capture. Females were sexed by beak color (females have a pink base, males have a blue base) and plumage (males have more iridescent plumage than females) (Witschi and Miller 1938). Females were blood sampled opportunistically throughout the breeding season at sequential reproductive stages; a) the first day after laying their first egg, b) at clutch completion (2 days after their final egg was laid), c) at day 8 of incubation, and d) between days 11-14 of chick rearing (N=36).

In fall, non-breeding birds were mist-netted on November 10, 2007 between 11:00-13:37 H. Several of the mist-netted females were recaptured adults (metal and color banded) from previous breeding seasons; however, these females were not sampled.

Therefore, we were at least mist-netting a mixed-age population with some adult birds. Additionally, because we mist-netted both males and females, we were able to confirm that mass did not vary with sex ($t = -1.83$, $P > 0.07$), so it is unlikely that mass was biased within our sampled females. Thus, we are confident our results were not biased by age or sex. Individuals were placed in cotton bags after capture, and processing time was staggered between 2-61 min post mist-netting (data not presented in this thesis). Time between capture and blood sampling did not affect mass, our index of body condition (mass/tarsus), or plasma leptin-like immunoreactivity ($P > 0.44$ in all cases), thus all mist-netted females were pooled for the current study. Sex was initially determined by eye ring pattern, with females characteristically exhibiting a pale outer eye-ring; however, this was subsequently shown not to be a reliable sexing criterion based on presence of ovaries or testes at dissection. Therefore, the only fall birds included in analysis were females sexed by dissection after euthanasia ($N = 21$).

An additional starling data set for the 1999-2001 breeding seasons (Williams and Vézina, unpubl.) was used for comparison of body mass, non-reproductive mass and fat mass with 2007 breeding season data to ensure that data from the 2007 breeding season was typical of other years and to examine plasma leptin-like immunoreactivity in relation to changes in fat mass in other years (not measured in 2007).

2.3.3 Plasma Leptin Assay

Plasma was analyzed for leptin-like immunoreactivity at the United States Department of Agriculture Laboratories, Baltimore, MD, USA, by Dr. John P. McMurtry using a chicken leptin radioimmunoassay (see below). Starling plasma dilutions are linear and parallel to leptin-like immunoreactivity levels in chicken plasma (Figure 2.1).

Additionally, previous work has shown that this chicken antibody does not cross-react with chicken insulin, glucagon or GLP-1. Samples were run in duplicate, and the intra-assay CV calculated from duplicate samples was 3.2%.

2.3.3.1 Leptin Radioimmunoassay

Putative recombinant chicken leptin (rcleptin) was kindly provided by A. Gertler (Raver et al. 1998). This preparation was used for the production of antisera, for radioiodinations, and as standards. Mouse and human recombinant leptin were purchased from Sigma Chemical Co., St. Louis, MO. The primary antibody, rabbit anti-rcleptin was purchased from a commercial source (Alpha Diagnostic International, San Antonio, TX).

2.3.3.2 Radioiodination

The iodination of rcleptin was conducted as previously described (McMurtry et al. 1994) with minor modifications. Aliquots of 10 μg of rcleptin were dissolved in 25 μl sodium phosphate buffer (0.5M, pH 7.5) and stored frozen at -80° C. For radioiodination, the vial was thawed and 0.7 mCI carrier-free I-125 sodium iodine (100 mCI/ml; Amersham Corp., Arlington Heights, IL) were added. Chloramine T (10 μg) was added in 10 μl sodium phosphate buffer (0.05M, pH 7.4), and the reaction was stopped after 1 minute by the addition of 20μg sodium metabisulphite (Fluka Chemical Corp., Ronkonhoma, NY) in 20 μl sodium phosphate buffer (0.05M). Un-reacted I-125-iodine was separated from I-125-labeled rcleptin by chromatography on Sephadex G-50 (Pharmacia, Piscataway, NJ) in 0.7 X 50 cm glass columns (Kontes, Vineland, NJ), pre-equilibrated with sodium phosphate buffer containing 1.0% bovine serum albumin, radioimmunoassay grade; Sigma Chemical Co). Putative recombinant chicken leptin was

iodinated to a specific activity of 50 Ci/g by this method. Following radioiodination, labelled rcleptin was aliquotted and stored at -80° C.

2.3.3.3 Assay Buffers and Conditions

Sodium phosphate buffer (0.05M phosphosaline, pH 7.4), containing 0.025M EDTA plus 0.05% Triton X-100 (Sigma Chemical Co) was used for dilution of primary and secondary antibodies. Standard hormone (putative recombinant chicken leptin) and tracer (radiolabeled rcleptin) were dissolved, diluted and stored in the phosphate buffer containing 1% BSA.

To enhance the sensitivity of the RIA, the assay was conducted under non-equilibrium conditions. On day 1, RIA diluent (100 µl) plus a similar volume of standard or plasma unknown were added to plastic tubes containing 100 µl of first antibody (rabbit anti-chicken leptin; 1:1600 working dilution), vortexed and incubated overnight at 4° C. On day 2, 100 µl tracer containing 6000 c.p.m. of I-125-labeled rcleptin was added to each tube, vortexed and incubated overnight at 4° C. On day 3, second antibody (100 µl of a 1:10 dilution of sheep anti-rabbit gamma globulin) and carrier (100 µl normal rabbit serum diluted 1:200 in phosphate buffer) was added to each tube, vortexed and incubated overnight at 4° C. Second antibody and normal rabbit serum were purchased from Linco, Inc., St. Charles, MO. On day 4, all tubes except the total count tubes were centrifuged at 2500 rpm, the supernatant aspirated, and the pellet counted in a gamma counter. All RIA data reductions were conducted using the log/logit transformation. Intra- and interassay coefficients of variation were calculated as described by Abplanalp et al. (1977).

2.3.4 Statistical Analysis

Data were analyzed in SAS 9.1 (SAS Institute Inc 2002). Only individuals for which we could obtain plasma leptin-like immunoreactivity values were included in the data set for analysis (N_{leptin}=57). Normality was evaluated using univariate analyses of raw data for correlations and regressions and using residuals for ANOVAs (Shapiro-Wilk). All of the analyzed traits approximated normality. Therefore, parametric tests were used for all analyses, including ANOVA General Linear Models (GLM) and subsequent LS means comparisons to evaluate variation in plasma leptin-like immunoreactivity, body mass, and body condition measures by stage (the Fall stage was included in all analyses excepting the analysis involving the Williams and Vézina dataset) and a GLM of plasma leptin immunoreacitivity with mass as a co-variate, Pearson Correlations of pooled stage residuals for plasma leptin-like immunoreactivity and both body mass and body condition, and a regression for plasma leptin-like immunoreactivity in relation to Julian date. All means are reported ± standard error. Additionally, P-values for all multiple comparisons of LS means were Tukey-Kramer adjusted.

2.4 Results

2.4.1 Variation in Body Mass and Condition

Body mass varied by stage in 2007 ($F_{4,51} = 24.35$, $P < 0.0001$; Fig. 2.2a); mass was highest during egg-laying (93.6 ± 1.9 g) and decreased by clutch completion (80.5 ± 1.3 g; $P < 0.0001$), and then did not vary significantly through to chick rearing. However, body mass in November (74.9 ± 0.9 g) was significantly lower than all stages of the breeding season ($P \leq 0.0053$ in all cases). Body condition (mass/tarsus) also varied by

stage ($F_{4,51} = 20.14$, $P < 0.0001$), and similar to body mass, decreased after egg-laying ($P < 0.0001$). Additionally, body condition in November was significantly lower than during egg-laying, at clutch completion and during incubation of the breeding season ($P \leq 0.0344$ in all cases), and almost significantly lower than during chick-rearing ($P < 0.055$).

To confirm that the mass and condition data during the 2007 breeding season were "typical" for European starlings during other breeding seasons, we compared the 2007 breeding season body mass data with pooled data from the 1999-2001 breeding seasons (Williams and Vézina unpubl. data, Fig 2.3; only breeding season data were available for these analyses). Analysis of variation in body mass by stage using the two data sets (2007 and 1999-2001) showed a dataset*stage interaction ($F_{3,215} = 5.08$, $P = 0.002$). This interaction occurred because although egg-laying and clutch completion body masses were not different by "year" ($P > 0.16$ in both cases), incubation ($P = 0.0221$) and chick rearing ($P = 0.0028$) body masses were higher in 2007 than in the 1999-2001 pooled breeding seasons. However, the patterns of decrease in body mass in the 1999-2001 pooled breeding seasons follow the same general patterns as in 2007. In 2007, body mass varied by stage ($F_{3,32} = 15.38$, $P < 0.0001$; Fig. 2.3); with body mass decreasing between egg-laying and clutch completion ($P < 0.0001$) and again between incubation and chick-rearing ($P < 0.0443$). Similar to the 2007 breeding season, in the 1999-2001 pooled breeding seasons, body mass also varied by stage ($F_{3,156} = 218.91$, $P < 0.0001$; Figs. 2.3, 2.4a), and decreased after egg-laying ($P < 0.0001$) and after incubation ($P = 0.007$).

We also analyzed the 1999-2001 pooled data for variation in non-reproductive body mass and fat mass to inform our interpretation of the 2007 plasma leptin-like immunoreactivity data. Non-reproductive mass varied by breeding stage ($F_{3,156} = 22.77$, $P < 0.0001$; Fig. 2.4a) and was highest during egg-laying and decreased by clutch completion ($P = 0.0011$) and again after incubation ($P = 0.0167$). Similarly, fat mass varied by stage ($F_{3,156} = 47.24$, $P < 0.0001$; Fig. 2.4b), again being highest during egg-laying (4% of non-reproductive body mass), decreasing by clutch completion (to 2% of non-reproductive body mass; $P < 0.0001$), but in contrast, remaining constant through to chick-rearing ($P > 0.79$).

2.4.2 Variation in Plasma Leptin-Like Immunoreactivity

Plasma leptin-like immunoreactivity varied by stage ($F_{4,52} = 21.11$, $P < 0.0001$; Fig. 2.2b). Plasma leptin-like immunoreactivity was not different between egg-laying and clutch completion, but then decreased significantly from clutch completion (23.8 ± 1.6 ng/mL) to incubation (13.0 ± 1.6 ng/mL) ($P = 0.0002$), remaining low during chick rearing. A small number of repeat sampled individuals confirmed this pattern (Fig. 2.5). However, plasma leptin-like immunoreactivity in non-breeding birds in November (23.7 ± 1.1 ng/mL) was significantly higher than in incubating and chick-rearing birds ($P < 0.0001$ in both cases), and not significantly different from values in egg-laying and clutch completion birds ($P > 0.53$ in both cases).

Over pooled stages (residuals controlling for stage), residuals for plasma leptin-like immunoreactivity were independent of both residuals for body mass ($r = -0.065$, $n = 56$, $P > 0.63$; Fig. 2.6a), and residuals for body condition ($r = 0.040$, $n = 56$, $P > 0.76$; Fig. 2.6b). Additionally, plasma leptin-like immunoreactivity still varied by stage when

controlling for body mass ($F_{4,50} = 21.00$, $P < 0.0001$); mass-corrected plasma leptin-like immunoreactivity still decreased significantly after clutch completion ($P = 0.0006$), and mass-corrected plasma leptin-like immunoreactivity in non-breeding females was still higher than in incubating and chick-rearing birds ($P \leq 0.0021$ in both cases).

We investigated whether variation in plasma leptin-like immunoreactivity was due to date rather than breeding stage *per se*. Plasma leptin-like immunoreactivity significantly decreased with date over the entire breeding season ($F_{1,34} = 43.38$, $r^2 = 0.56$ (Adj = 0.55), $P < 0.0001$); however, within each of the breeding stages, sampling date did not affect plasma leptin-like immunoreactivity ($P > 0.11$ in all cases).

2.5 Discussion

In this study, we report for the first time in a free-living avian species that plasma leptin-like immunoreactivity varies with reproductive stage and season in female European starlings sampled opportunistically during the breeding season and in non-breeders in November. Plasma leptin-like immunoreactivity was highest during egg-laying and clutch completion, significantly decreased during incubation, and remained low during chick rearing. A small number of repeat sampled individuals confirmed this pattern. This variation in plasma leptin-like immunoreactivity was consistent with a functional role of putative leptin in ovarian function and egg production (see below). However, we also found that plasma leptin-like immunoreactivity was significantly higher in November than during incubation and chick rearing, and not different from levels at egg-laying and clutch completion, indicating a seasonal change in leptin-like immunoreactivity. Although there was marked and consistent variation in total body mass and fat mass with breeding stage and season in this population, patterns of variation

in plasma leptin-like immunoreactivity did not parallel changes in body mass or composition. Thus, changes in plasma leptin-like immunoreactivity throughout the 2007 breeding season did not coincide with body mass changes in 2007 or with the typical timing of percent fat mass changes (evidenced by 1999-2001 data). For example, body mass, body condition, and fat mass decreased between egg-laying and clutch completion; whereas, plasma leptin-like immunoreactivity did not decrease until between clutch completion and incubation (when body mass did not change). Similarly, plasma leptin-like immunoreactivity was high in non-breeding birds in November, at a time when both body mass and body condition were lower than at any stage of the breeding season. Furthermore, neither body mass nor body condition was correlated with plasma leptin-like immunoreactivity (residuals of individuals controlling for stage) and mass-corrected plasma leptin-like immunoreactivity showed the same strong seasonal or stage-specific pattern of variation. Therefore, we conclude that among free-living female European starlings, variation in plasma leptin-like immunoreactivity does not reflect changes in body mass or fat mass but is instead associated with breeding stage or seasonal variation itself.

While we found no evidence for a robust link between plasma leptin-like immunoreactivity and body mass, body condition, or percent body fat, in contrast to our findings, other studies have shown a strong relationship between plasma leptin-like immunoreactivity and body mass or percent body fat in mammals (Halaas et al. 1995, Considine et al. 1996) and in captive or domesticated quail and chickens (Lohmus et al. 2006, Shi et al. 2006). This could be mediated through food intake: several studies have found that administration of recombinant mammalian and putative chicken leptins in

birds decreases food intake in great tits, Japanese quail, and chickens (Denbow et al. 2000, Lohmus et al. 2003, Kuo et al. 2005, Lohmus et al. 2006). Indeed, immunization against purported recombinant chicken leptin results in increased food intake, weight gain, and fat deposition in chickens (Shi et al. 2006). However, we suggest that the contrast between these previous studies and our study of free-living birds is due to differences in the range of body masses in the respective species. While there is an extensive range of body masses and adiposity in many mammalian species as well as poultry species, the range of body fat among free-living starlings is extremely narrow by comparison. For example, humans display an enormous range of percent body fat, and one study that correlated plasma leptin to percent body fat displayed a body fat range of approximately 5-65 % (Considine et al. 1996). Similarly, the difference in abdominal fat mass between putative recombinant chicken leptin immunized hens (115.6 g) and control hens (78.0 g) in one study was 37.6 g, which means the treated hens had 50 % more abdominal fat mass than the control hens, illustrating a large possible range in abdominal fat mass among captive hens (Shi et al. 2006). In contrast, the largest decrease in total percent lipid mass during the reproductive season in starlings was only from 4% to 2% between egg-laying and clutch completion (Williams and Vézina, unpubl.), and total body mass varies from only 70-100g. Thus, any correlation between plasma leptin-like immunoreactivity and body fat would have to be extremely high to be detected in a free-living bird that has such little variation in body fat. Furthermore, because leptin has many functional roles besides adiposity signalling, including reproduction and immune function, and may vary seasonally (see Introduction and Chapter 1), it is not surprising

that we did not detect a relationship between plasma leptin-like immunoreactivity and body mass or extrapolated percent body fat in our study.

Our results indicate that, independent of body mass effects, elevated plasma leptin-like immunoreactivity was associated with the egg-laying (continuing through clutch completion) phase of breeding, and that there was also a seasonal shift in plasma leptin-like immunoreactivity, with elevated plasma levels in non-breeding birds in November. While several studies have demonstrated seasonal variation in serum leptin among some captive mammals including blue foxes, woodchucks and European brown bears (*Ursus arctos arctos*) (see Introduction; Hissa et al. 1998, Concannon et al. 2001, Nieminen et al. 2001, Nieminen et al. 2002, Mustonen et al. 2005), these relationships remain unexplored in birds. Thus, comparisons between our data on reproductive stage and seasonal changes in plasma leptin-like immunoreactivity and other studies on free-living birds are not possible at this time. However, we suggest that the elevated plasma leptin-like immunoreactivity that we found in egg-laying and non-breeding birds most likely have different functional explanations.

Our finding that plasma leptin-like immunoreactivity varied with breeding stage, and was highest at a time of increased ovarian function is consistent with the link between leptin and reproduction, which has been previously reported in mammals and birds (see Introduction). Some studies have examined the relationship between putative plasma leptin and reproduction in birds and demonstrated that recombinant human and purported chicken leptin administration can alter reproductive steroid secretion, including estradiol, can change ovarian cell proliferation and apoptosis, and can even advance puberty in chickens (Paczoska-Eliasiewicz et al. 2003, Paczoska-Eliasiewicz et al. 2006,

Sirotkin and Grossmann 2007, Sirotkin et al. 2007). In addition, immunization against purported recombinant chicken leptin has been shown to decrease laying rate among hens (Shi et al. 2006). Finally, the liver is the primary location of lipogenesis, and in particular, vitellogeneis, during egg production in birds, which could provide rational for hepatic expression of purported leptin in birds, unlike mammals (Taouis et al. 1998, Ashwell et al. 1999b, Dridi et al. 2005, Kochan et al. 2006). The pattern of elevated plasma leptin-like immunoreactivity during egg-laying that we found in the breeding season appears to follow fluctuations previously shown in plasma estradiol in free-living starlings by Dawson (1983), perhaps indicating that changes in plasma leptin-like immunoreactivity may be linked to variation in plasma estradiol during the breeding season. In wild European starlings, plasma estradiol (E2) significantly increases above winter concentrations beginning in pre-breeding (March), peaks at the beginning of the breeding season during egg formation, decreases during egg laying, and tends to decrease at incubation and at chick rearing, with concentrations being significantly lower post chick rearing compared to incubation (Dawson 1983). Furthermore, Williams et al. (2004) demonstrated that in free-living European starlings, plasma E2 increases quickly during rapid yolk development in pre-laying and was elevated in laying birds until they no longer had any yolky follicles, which occurred prior to clutch completion. Additionally, in captive female canaries (*Serinus canaria*), 17 β-estradiol peaks 1 day prior to the commencement of egg-laying, decreases throughout laying, then drops significantly (three-fold) further the first day of incubation, which in this species may be before, after, or the same day as ovulation of the last egg (Sockman and Schwabl 1999).

In contrast to the possible relationship between elevated plasma leptin-like immunoreactivity and estradiol during egg-laying, high plasma leptin-like immunoreactivity in fall could result from other causes. Highly energetic activities such as foraging and thermogenesis burn fat stores, and because many studies have shown that serum leptin concentrations positively correlate to the quantity of adipose tissue (e.g. Considine et al. 1996), one would predict low leptin-like immunoreactivity levels among the Fall sample starlings in this study. However, we found high plasma leptin-like immunoreactivity in these birds. Similar to the breeding birds, it appears as if body mass does not reflect plasma leptin-like immunoreactivity levels in the fall sampled birds. Perhaps these high leptin-like immunoreactivity levels could be due to a positive relationship with thermogenesis in starlings in fall. However, there is some evidence to suggest a negative relationship between leptin and thermogenesis; in rats acclimated to cold temperatures, leptin administration decreased both food intake and thermogenesis (heat production) in brown adipose tissue (Abelenda et al. 2003). Additionally, in non-reproductive Brandt's voles (*Lasiopodomys brandtii*) exposed to cold temperatures, serum leptin levels decreased (Zhang and Wang 2007a). Similarly, in wild Mongolian gerbils *(Meriones unguiculatus*), serum leptin levels are lower in the winter than during the other seasons (Zhang and Wang 2007b). There is no research available on the effects of cold temperatures on putative avian leptin among captive or free-living birds. Thermogenesis is just one possible avenue of investigation into this relationship; perhaps these elevated levels could even reflect seasonal changes in immune function or another relationship entirely. Research must be conducted to explore possible explanations and mechanisms for elevated plasma leptin-like immunoreactivity among free-living starlings

in fall and winter to seek to explain why these levels would be elevated during cold temperatures among birds with low body mass and fat.

Because plasma leptin-like immunoreactivity appears to vary similarly to plasma estradiol during the breeding season in female European starlings, in a subsequent study, we performed a manipulation on captive starlings to determine if exogenous estradiol and/or photoperiodic stimulation of the reproductive system induce elevations in plasma leptin-like immunoreactivity in order to investigate whether the elevated plasma leptin-like immunoreactivity we observed among free-living starlings in the breeding season could be related to the surge of estrogen produced for egg-formation (Chapter 3).

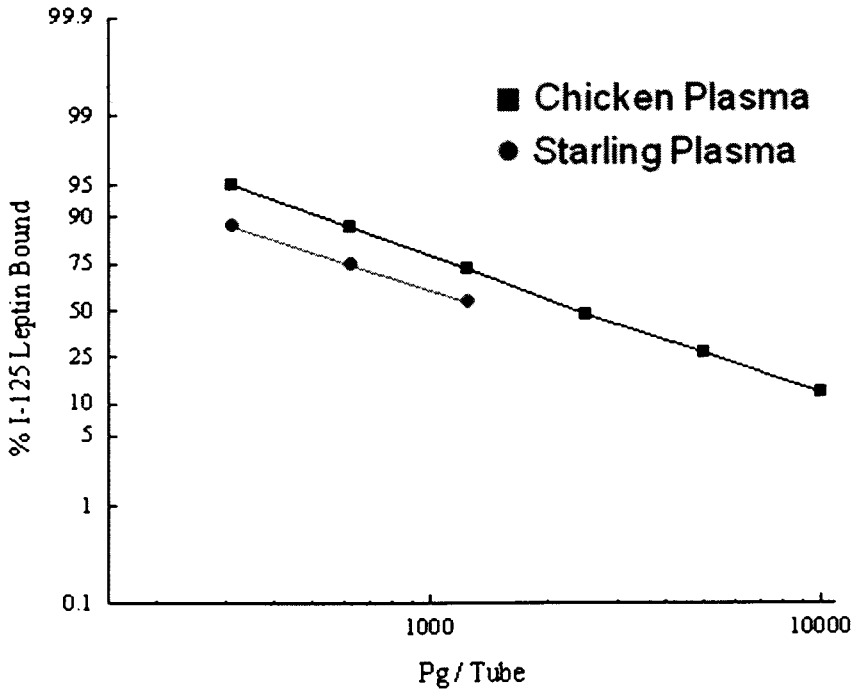

Figure 2.1 **Dilution curve of plasma leptin-like immunoreactivity for chicken and European starling (*Sturnus vulgaris*). Starling plasma dilutions are linear and parallel to leptin-like immunoreactivity levels in chicken plasma.**

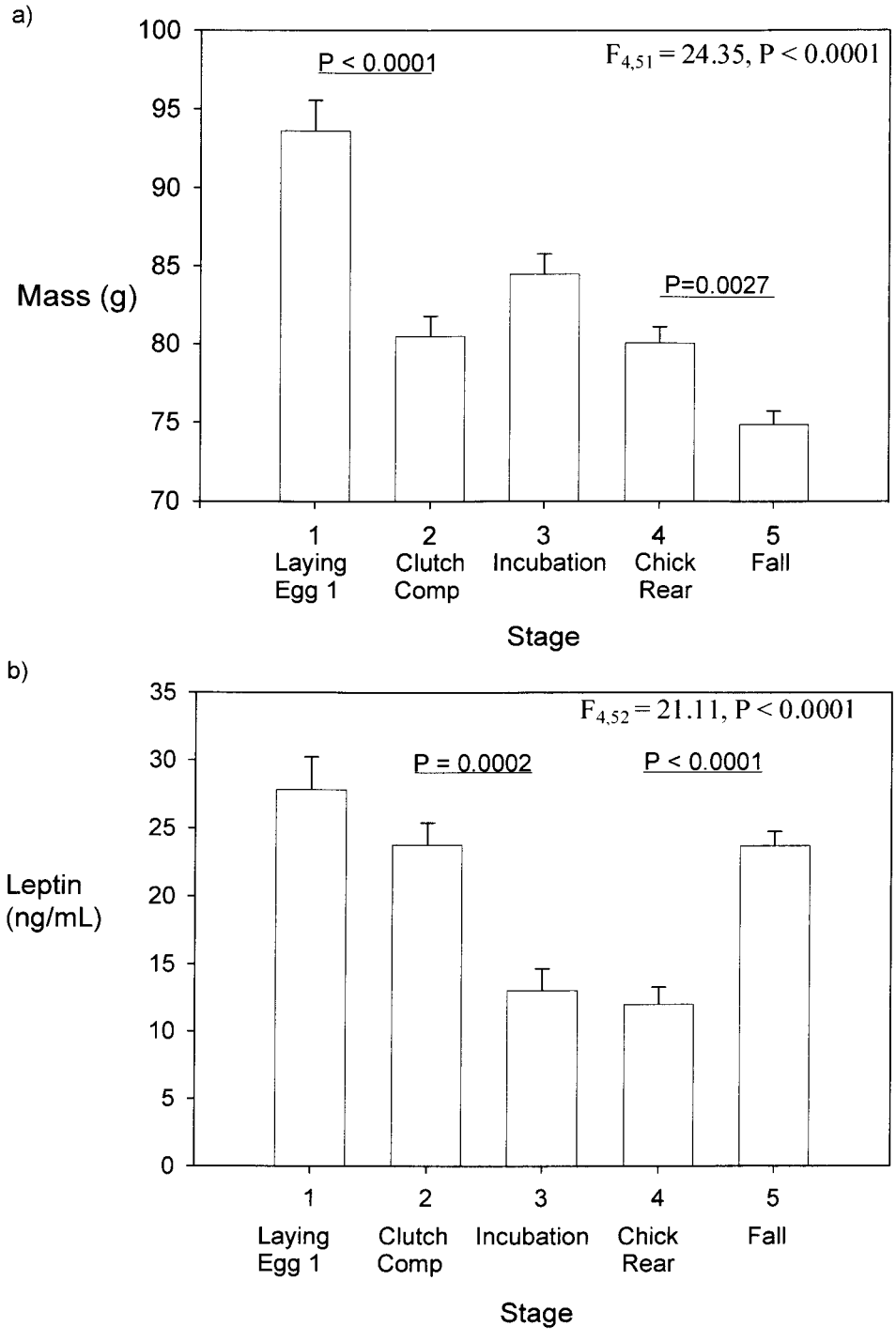

Figure 2.2 **Variation in (a) body mass (g) and (b) plasma leptin-like immunoreactivity (ng/mL) by stage (breeding and Fall) among free-living female European starlings (*Sturnus vulgaris*).**

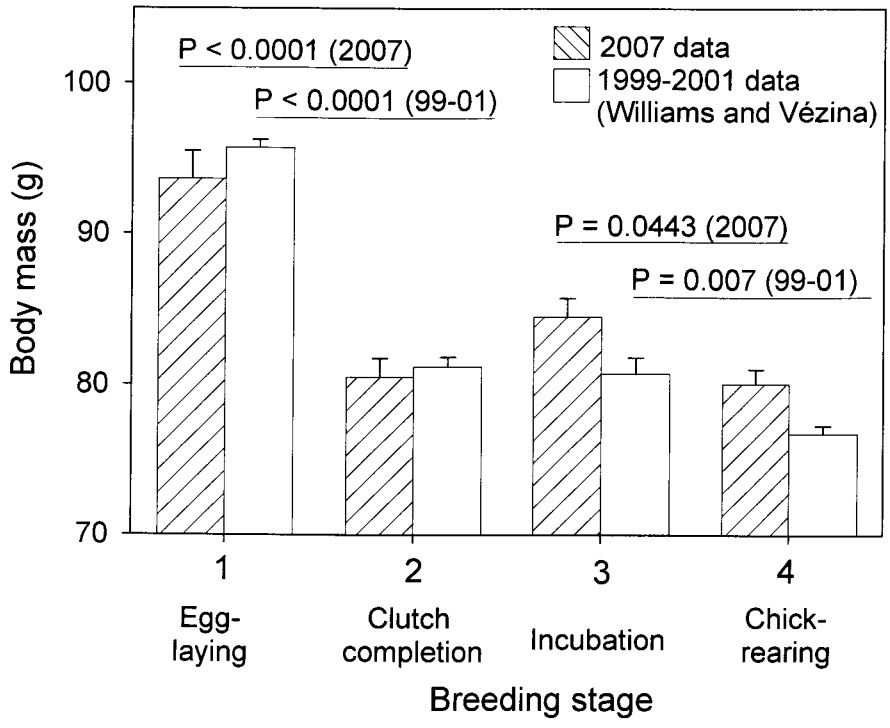

Figure 2.3 **A comparison of free-living female European starling (*Sturnus vulgaris)* body mass data (g) by breeding stage in the 2007 breeding season ($F_{3,32} = 15.38$, $P < 0.0001$) and the pooled 1999-2001 breeding seasons ($F_{3,156} = 218.91$, $P < 0.0001$).**

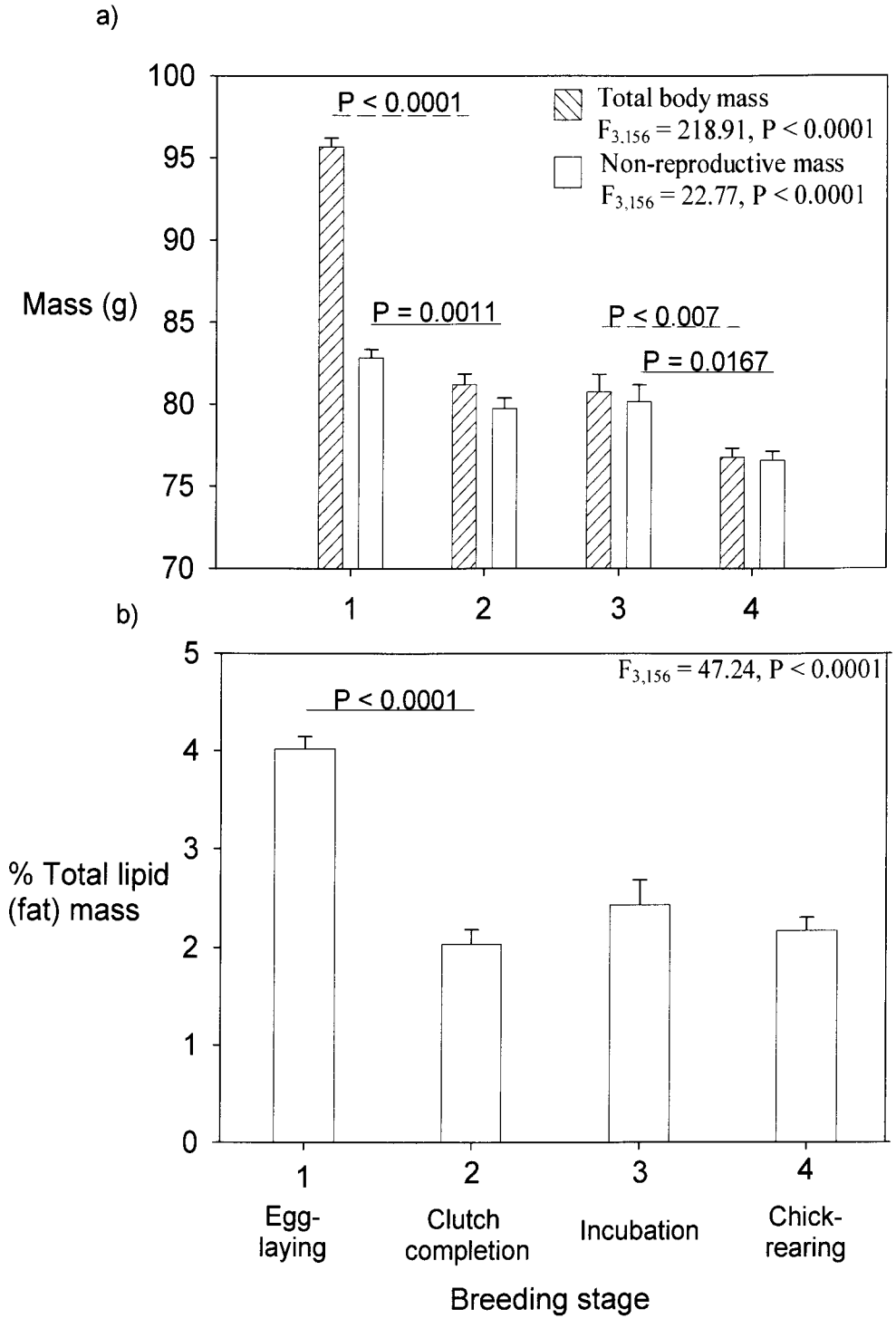

Figure 2.4 **Pooled data from free-living female European starlings (*Sturnus vulgaris)* during the 1999-2001 breeding seasons (Williams and Vézina, unpubl.): (a) Total body mass and non-reproductive mass (g) by stage; and (b) % total fat mass by stage.**

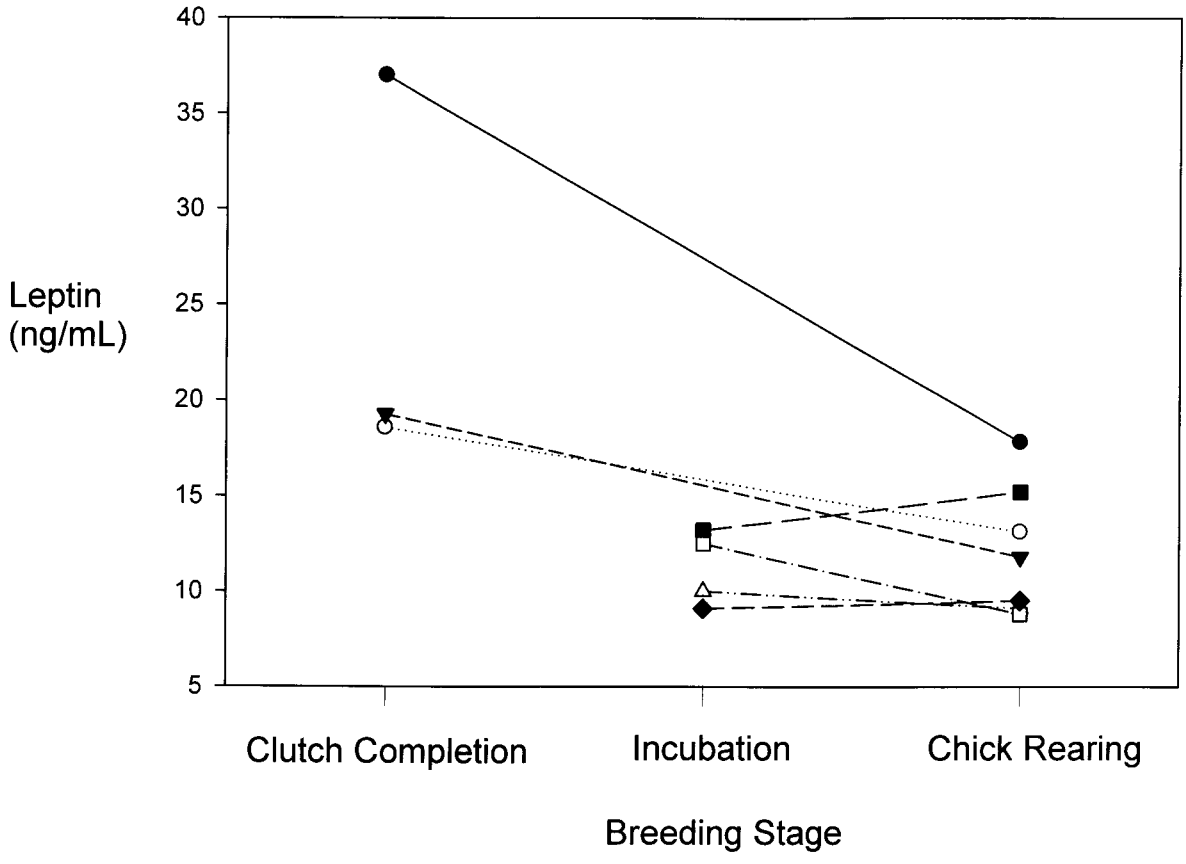

Figure 2.5 **Plasma leptin-like immunoreactivity values (ng/mL) for 7 repeat sampled free-living female European starlings (*Sturnus vulgaris)* during the 2007 breeding season by stage.**

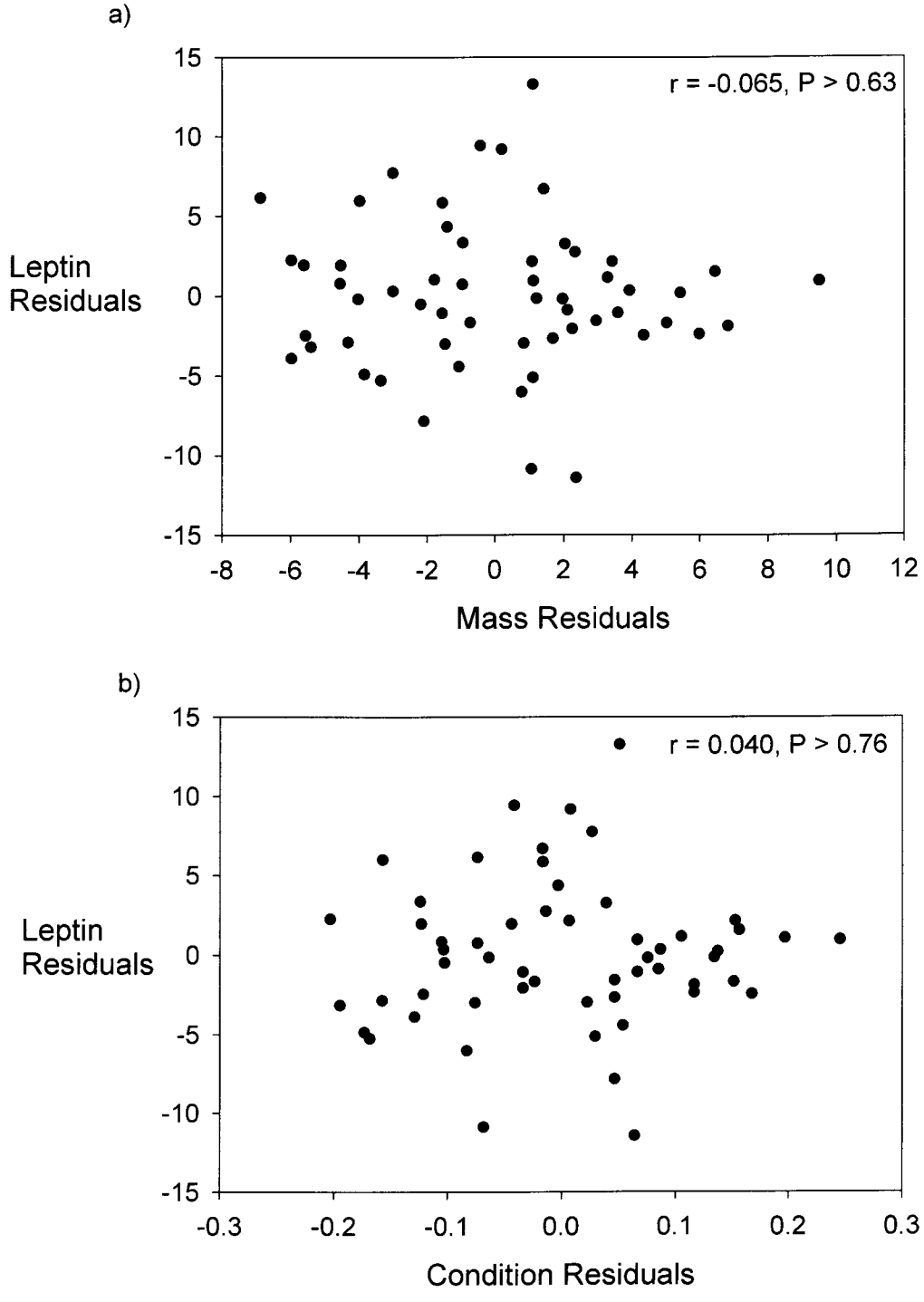

Figure 2.6 **Correlations of (a) residuals of plasma leptin-like immunoreactivity with residuals of mass when controlling for stage; and (b) and residuals of plasma leptin-like immunoreactivity with residuals of condition (mass/tarsus) when controlling for stage among free-living female European starlings (*Sturnus vulgaris)* during the 2007 breeding season. Individuals pooled across breeding stages and fall (n = 56).**

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Chapter 3 :
DOES PHOTOPERIOD OR ESTROGEN REGULATE LEPTIN-LIKE IMMUNOREACTIVITY IN FEMALE EUROPEAN STARLINGS (*STURNUS VULGARIS*)?

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3.1 Abstract

Leptin has been shown to have wide-ranging effects in mammals, including fat regulation, immune function, and reproduction. Additionally, plasma leptin has been shown to vary seasonally and may be linked to photoperiod and gonadal responsiveness to photostimulation. While the roles of putative leptin in birds appear to be similar to functions in mammals, research on purported avian leptin remains controversial, and the relationship between plasma leptin-like immunoreactivity and photoperiodic stimulation in birds remains unknown. However, there is evidence to suggest that purported leptin effects the secretion of avian reproductive hormones including estrogens. In a previous study on European starlings (*Sturnus vulgaris*), we found high plasma leptin-like

immunoreactivity during egg laying that decreased at incubation and remained low during chick rearing. In the current experimental study, we investigated the association between plasma leptin-like immunoreactivity, day length and reproduction to determine whether the high leptin-like immunoreactivity we observed during egg-laying in free-living starlings could be caused by elevated plasma estradiol (E2) - which has been shown to increase during egg-formation - and/or photoperiodic stimulation. Photo-sensitive female European starlings were captured in November and placed on a non-stimulatory short day length (SD 8L:16D) and injected with 17 β-estradiol (E2, at physiological levels) for five consecutive days. Then females were transferred to a stimulatory long day length (LD 16L:8D) and again injected with E2 for five consecutive days. Though the E2 injections effectively increased plasma E2 levels, there was no E2 or photoperiodic effect on plasma leptin-like immunoreactivity. There were, however, other treatment effects for females on LDs: 1) oviduct mass exhibited a positive dose-dependent response to E2, though ovary and yolky follicle development were not stimulated; and 2) plasma vitellogenin (VTG) displayed a positive dose dependant response to E2, although plasma very low density lipoprotein (VLDL) levels did not respond. Because photoperiodic and E2 stimulation did not result in full reproductive development, it remains possible that elevated plasma leptin-like immunoreactivity is related to later stages of reproductive development, such as folliculogenesis and rapid yolk development. Therefore, we cannot conclusively determine whether photostimulation and elevated plasma E2 cause elevation of plasma leptin-like immunoreactivity during egg-production in wild birds.

3.2 Introduction

Leptin is a protein hormone product of the obese (ob) gene that was first sequenced and cloned in mice and humans relatively recently (reviewed in Zhang et al. 1994), and has been shown to have a wide range of physiological roles in mammals including: feeding regulation, adiposity signalling, reproduction, and immune function (reviewed in Friedman and Halaas 1998, Chehab 2000, La Cava and Matarese 2004, reviewed in Budak et al. 2006). Leptin has been less extensively studied in birds, and data is restricted to captive individuals or domesticated species (but see Kochan et al. 2006) (see Chapters 1 and 2 for more background on this issue). Avian studies have measured variation in plasma leptin-like immunoreactivity by using heterologous leptin radioimmunoassays (RIAs; based on the purported recombinant leptin protein derived from the reported avian gene sequence) or have performed manipulations using mammalian leptin or putative recombinant chicken leptin. Several avian studies have documented similarly diverse physiological effects of mammalian leptins or putative chicken leptin in birds consistent with those reported in mammals, including effects on reproduction, immune function, body mass and involvement in feeding regulation (Denbow et al. 2000, Lohmus et al. 2003, Lohmus et al. 2004, Kuo et al. 2005, Lohmus et al. 2006, Alonso-Alvarez et al. 2007, Sirotkin and Grossmann 2007, Sirotkin et al. 2007).

Some studies on captive mammals have shown seasonal variation in plasma leptin (Hissa et al. 1998, Concannon et al. 2001, Nieminen et al. 2001, Nieminen et al. 2002, Mustonen et al. 2005), although the key factors regulating this seasonal variation are poorly understood. Plasma leptin is affected by changes in day length or photoperiod in

several mammalian species. For example, serum leptin is elevated during long day lengths (16L:8D) relative to short day lengths (6L:18D or 8L:16D or 10L:14D) in Siberian or Djungarian hamsters (*Phodopus sungorus*) (Atcha et al. 2000, Horton et al. 2000, Klingenspor et al. 2000) and Soay rams (*Ovis aries*) (Marie et al. 2001). Drazen et al. (2000) examined photoperiod in the context of gonadal regression among male Siberian hamsters and found that hamsters with regressed gonads that were housed on a short day length had low leptin levels, whereas short day individuals with functional gonads had high leptin, similar to hamsters housed on a long day length (with functional gonads). These studies clearly illustrate that plasma leptin levels can be related both to photoperiod and to gonadal responsiveness to photostimulation in mammals, but similar data are not available for birds.

Leptin has also been shown to be involved in many aspects of reproduction in mammalian (Henry et al., 2001; Steiner et al., 2003; reviewed in Budak et al., 2006) and avian species; including regulation of puberty, ovarian function, and gonadal steroid production in birds (Paczoska-Eliasiewicz et al. 2003, Paczoska-Eliasiewicz et al. 2006, Shi et al. 2006, Sirotkin and Grossmann 2007, Sirotkin et al. 2007). Leptin receptors have been identified in the ovary (Paczoska-Eliasiewicz et al. 2003), and injections of leptin or culturing of ovarian cell walls or granulosa cells with human recombinant leptin have both been shown to effect the secretion of several reproductive steroids and to change cell apoptosis and cell proliferation in chickens (Sirotkin and Grossmann 2007, Sirotkin et al. 2007). In addition, daily injections of purported recombinant chicken leptin in pullets fed *ad libitum* resulted in advanced pre-pubertal increases in plasma LH, progesterone and estradiol, and advanced puberty (as indicated by first oviposition)

which the authors attributed to the enhancement of folliculogenesis and attenuation of ovarian apoptosis (Paczoska-Eliasiewicz et al. 2006). Putative leptin is also expressed in the liver in birds, unlike mammals, perhaps because the liver is the major site of lipogenesis (Taouis et al. 1998, Ashwell et al. 1999b, Dridi et al. 2005, Kochan et al. 2006), and specifically vitellogenesis, during egg production.

In a previous study (see Chapter 2) we described seasonal variation in plasma leptin-like immunoreactivity in free-living European starlings (*Sturnus vulgaris*). We found that plasma leptin-like immunoreactivity was elevated during early stages of reproduction (egg-laying) relative to later stages of reproduction (incubation and chick rearing), and also in Fall when day length was short relative to the breeding season. Here we use an experimental study of captive female European starlings to investigate this association between plasma leptin-like immunoreactivity, day length and reproduction. Specifically we test the hypothesis that elevated plasma leptin-like immunoreactivity is caused by a) seasonal, photoperiodic (long day length-dependent) reproductive development, and/or b) elevated estrogen required for egg production. Wild-caught photo-sensitive female European starlings were maintained on a short non-stimulatory day length (SD 8L:16D) and then transferred to a stimulatory long day length (LD 16L:8D) and were either injected with 17 β-estradiol (E2), sham-treated or non-manipulated during both photoperiods. Birds were blood sampled for measurement of plasma leptin-like immunoreactivity, plasma estradiol and yolk precursors and were sacrificed to obtain data on reproductive development. Thus, this experimental design allowed us to assess the differential effects of photoperiod and exogenous E2 on plasma

levels of leptin-like immunoreactivity and reproductive measures, including E2 and yolk precursors.

3.3 Methods

3.3.1 Collection of Female European Starlings

European starlings were mist-netted at Davistead Dairy Farm in Langley, British Columbia (49°10'N, 122°50'W) on November 10, 2007. Birds were sexed, measured, weighed (± 0.1 g) and a blood sample was taken at capture. Sex was initially determined by eye ring pattern, with females characteristically exhibiting a pale outer eye-ring. All birds were subsequently sexed based on presence of ovary or testes after dissection, which showed that the presence or absence of an eye ring was not a reliable sexing criterion. Dissection enabled reliable sexing, and thus only females were used in analyses. Females (n = 21) were taken into captivity and were held in the Simon Fraser University Animal Care Facility until December 21 when all birds were euthanized at the end of the experiment. All experimental procedures were conducted following Canadian Council of Animal Care (CCAC) guidelines in accordance with Simon Fraser University Animal Care Committee (UACC) permit number 829B-96.

3.3.2 Animal Husbandry and General Experimental Protocol

Birds were initially transferred to a short day light cycle (SD; 8L:16D) which mimics their natural winter light cycle (on the day of capture, sunrise was at 7:12 PST and sunset at 16:36 PST resulting in a day-length of 9 hrs 24 min). Birds were held in metal cages (103 x 40.5 x 35.5 cm) with 4-5 birds per cage and were provided with water and Roudybush Daily Maintenance (Roudybush) food *ad libitum*. Body mass (± 0.01g)

was recorded 48 and 96 hours post-capture to determine when mass stabilized prior to the start of experiments. Body mass did not change with transfer to captivity; there was no time effect on individual body mass (Repeated Measures ANOVA, Wilks' Lambda, $F_{2,16} = 0.08$, $P > 0.92$). All blood samples during the study were collected from the right or left brachial vein between (between 9:00-13:00 PST) within < 3 min of handling, the samples were centrifuged and hematocrit was recorded, and plasma was frozen at -20°C within 5 hours of sampling. All samples were assayed for plasma leptin immunoreactivity and plasma triglyceride, while VTG and E2 were only assayed at select sampling periods.

Once female body mass had stabilized, birds were used in three sequential experiments: a) after seven days of captivity and transfer to SD (8L:16D), birds were blood sampled to determine circadian patterns of plasma leptin and triglyceride (data for this experiment are not reported in this thesis); b) seven days after the conclusion of the circadian study (16 days post-capture), the birds began the Short Day Photoperiod and E2 Treatment Experiment, and c) six days after the switch to a long day light cycle (LD; 18L:6D), birds began the Long Day Photoperiod and E2 Treatment Experiment. The experimental design for experiments (b) and (c) is presented in Fig. 3.1.

3.3.3 Preparation of Estradiol Solution

The E2 solution was prepared by dissolving 17β-estradiol (E2) into diethyl ether and then perfusing the ether through canola oil with air. The putative dose of E2 in the canola oil solution was 1 ug/g for a 90 g bird. Williams (1999b) injected daily E2 doses of 1.2 ug/g, and Vezina et al. (2003) administered daily E2 doses of 1.5 ug/g in zebra finches (*Taeniopygia guttata*) to produce hormonal responses within the physiological

range. To validate our E2 solution, we injected 30 μL of the E2 solution into eight female Zebra Finches for five consecutive days, and on 1 day post-final injection we analyzed the plasma for triglycerides (plasma VLDL). Plasma triglyceride levels were significantly elevated in E2-treated zebra finches (16.3 ± 3.3 mg/mL) compared to non-breeding birds (4.7 ± 3.3 mg/mL, $P < 0.0001$), and were not significantly different to mean plasma levels in egg-laying zebra finches (18.7 ± 2.2 mg/mL) ($P > 0.99$).

3.3.4 Effects of Short Day Photoperiod and Estradiol Treatment

After 15 full days in captivity on the 8L:16D light cycle, one day prior to the first day of E2 injection, birds were randomly divided into three treatment groups: a) non-manipulated females (n = 7) which received no daily injections of E2 or vehicle and were only handled for weighing, b) vehicle-injected females (n = 6) which received five daily 50 μL i.m. injections of canola oil into pectoral muscle, and c) E2-injected females (n = 8) which received 5 daily 50 μL i.m. injections of the E2 solution (1 μg/g). Injections were administered between 0900-1100 PST. Body mass was recorded one day prior to the first day of injection, and on day 1 and day 7 after the last E2 injection, and birds were blood sampled on day 1 of the E2 treatment prior to injections, and again on days 1 and 7 after the last injection.

3.3.5 Effects of Long Day Photoperiod and Estradiol Treatment

All females were transferred from short days (8L:16D) to a long day (LD) light cycle of 18L:6D nine days after the last E2 injection in the SD experiment. After six days on 18L:6D, birds were injected with either vehicle or one of two E2 doses. Individuals in the non-manipulated group from the SD experiment were randomly

distributed into two vehicle groups to receive injections of either 50 μL (n = 3) or 100 μL (n = 4) of canola oil in the LD experiment (matching the volume of vehicle in E2-treated females). However, there were no significant differences for any measured traits in relation to volume of vehicle injected ($P > 0.38$ in all cases; Table 3.1); therefore, subsequently these birds were pooled into a single vehicle-only group (n = 7). Individuals in the E2- and vehicle-injected groups from the SD experiment were both split randomly into two groups, a) E2-injected females (n = 7) which received 5 daily 50 μL i.m injections of the E2 solution (1 μg/g), and b) E2-injected females (n = 7) which received 5 daily 100 μL i.m. injections of the E2 solution (2 μg/g). This allowed us to test the effect of pre-exposure to E2 during short days on the response to subsequent E2 treatment on long days (though sample sizes were small). Injections were administered between 0900-1100 PST. Body mass was recorded one day prior to the first day of E2-injection, and again one day after the last injection, and birds were blood sampled on day 1 of E2-treatment prior to injections, and again one day after the last injection (on day 12 of the 18L:6D light cycle).

Birds were euthanized by exsanguination with anaesthesia one day after the last E2 injection i.e. day 12 of 18L:6D. Dawson and Goldsmith (1983) showed that in female European starlings, photoperiod-dependent reproductive function (plasma LH and FSH levels) peaked two weeks after transfer from 8L:16D to 18L:6D, and that birds then rapidly became photorefractory. Therefore, we assumed that the gonadal, oviduct and yolk precursor measurements for our females were obtained at the peak of the LD photoperiodic response. After euthanasia, we removed the ovaries and oviducts by

dissection and recorded wet mass (± 0.0001 g). Oviducts were frozen at -20° C for 3 weeks, before being lyophilized for three days, to obtain tissue dry mass (± 0.0001 g).

3.3.6 Plasma Leptin Assay

Plasma was analyzed for leptin-like immunoreactivity at the United States Department of Agriculture laboratories, Baltimore, USA by Dr. John P. McMurtry using a putative chicken leptin radioimmunoassay (see Chapter 2). Samples were run in duplicate, and the intra-assay CV calculated from duplicate samples was 3.2%.

3.3.7 Plasma Estradiol Assay

Plasma E2 determinations were conducted in Dr. Wynne-Edwards' Laboratory at Queen's University, Kingston, Ontario, Canada. Following C18 solid phase extraction, the concentration of 17β-estradiol (E2) in each starling plasma sample was determined using a commercially available enzyme-linked immunosorbent assay (Ecologiena, Japan Enviro Chemicals Ltd., Abraxis LLC, Warminster, PA, USA). Plasma samples were extracted using C18 columns (CUC18156, United Chemical Technologies, Chromatographic Specialties Inc., Brockville, ON, Canada) following the procedure described in Williams et al. (2005) and Wagner et al. (2008). Prior to the solid phase extraction procedure, 50 µL of starling plasma was added to 1 mL of distilled/de-ionized water (dd). Using a vacuum, each column was primed with 3 mL of HPLC-grade methanol, followed by 10 mL of dd water, followed by the entire diluted plasma sample, and then washed with 10 mL of dd water. E2 was eluted from the column with 5 ml of 80% methanol into 12 mm x 75 mm borosilicate culture tubes (47729-570, VWR,

Mississauga, ON, Canada). Each sample was then evaporated to dryness under vacuum and reconstituted in 300 μL of 10% methanol.

Assay standards and extracted plasma samples were assayed in duplicate. In an uncoated microplate, 100 μL of the standard or the reconstituted sample (equivalent to 16.7 μL of the original sample) were mixed with 100 μL of enzyme conjugate solution. Then 100 μL of this mixture was transferred to an antibody-coated microplate. The antibody-coated microplate was incubated for 1 hour at room temperature. The microplate was then washed 4 times using a plate washer (Skanwasher 400, Molecular Devices, Sunnyvale, CA). Following the addition of 100 μL of colour solution, the microplate was incubated for 30 minutes to allow for colour development. Samples with a coefficient of variability (CV) that exceeded 15% were repeated as a singlet on another plate and used to reject one of the two original determinations. Two levels of controls at 143 pg/mL and 455 pg/mL, yielded an intra-assay CV of 10.1% and 1.8% and inter-assay CV of 13.6% and 7.5%, respectively.

A pool of starling plasma was used to quantify the recovery of E2. A starling plasma pool was extracted in parallel over two C18 columns, quantified in duplicate by immunoassay and re-quantified as a singlet on a subsequent plate. Aliquot 1 read at 8.3 pg/well (CV 5.8%) and aliquot 2 read at 6.7 pg/well (CV 5.2%) yielding a CV of 13.9% for replicate extraction. One aliquot of the pool was spiked by 16.7 pg/well before extraction. This spiked sample was quantified in duplicate at 10.9 pg/well (CV=4.2%).

3.3.8 Plasma Yolk Precursor Assays

Plasma samples were assayed for yolk precursors using vitellogenin-zinc and total triglycerides as indices of vitellogenin and very low density lipoprotein respectively

(following Mitchell and Carlisle 1991). These assays have been widely used in yolk precursor studies of numerous avian species (Williams 1999b, Caro et al., Gorman et al.) including European starlings (Christians and Williams 1999a, 1999b, Challenger et al. 2001, Vezina and Williams 2003). We assayed plasma triglyceride in a colorimetric endpoint assay using free glycerol reagent (F6428 Sigma Chemicals, St. Louis, MO) and triglyceride reagent (T2449) and calculated individual triglyceride levels as (total glycerol - free glycerol). Samples were run in duplicate, and samples were re-assayed if the intra-assay coefficient of variation exceeded 15%. Inter-assay coefficients of variation for free glycerol and total glycerol were 8.4% (n = 8) and 3.5% (n = 8) respectively, and average intra-assay coefficients of variation were 7.6% (n = 23) and 9.5% (n = 23) respectively. Plasma vitellogenic zinc was assayed using the zinc kit (Wako Chemicals, Richmond, VA) and vitellogenic zinc was calculated by subtracting non-vitellogenic (albumin-bound) zinc from total plasma zinc (see Williams 1999b for further details). Samples were run in duplicate, and samples were re-run if intra-assay % CV exceeded 10%. The inter-assay coefficient of variation for the VTG-Zn assay was 6.3% (n = 9) and the intra-assay CV was 5.4% (n = 7).

3.3.9 Statistical Analysis

Data were analyzed in SAS 9.1 (SAS Institute Inc 2002). Plasma leptin-like immunoreactivity, hematocrit, body mass, and ovary mass approximated normality; however, the other measured traits were log transformed to achieve normal distributions. Plasma triglyceride was log transformed for correlations; however, the data were not transformed for ANOVAs or Paired T-Tests because the residuals approximated normality (in contrast, the triglyceride data for the zebra finch E2 validation was log

transformed for the ANOVA because the residuals were not normal). VTG was log transformed for correlations and ANOVAs (untransformed residuals not normal). Similarly, oviduct mass and plasma E2 were both log transformed for correlations and ANOVAs. Parametric tests were used for all analyses. Firstly, a General Linear Model (GLM) was performed on capture body mass, hematocrit, plasma triglyceride, and plasma leptin-like immunoreactivity to ensure that treatment group assignments were unbiased and random. Next, Repeated Measure ANOVA GLMs were performed for mass, hematocrit, plasma leptin-like immunoreactivity and plasma triglyceride on short days (at pre-injection, 1 day and 7 days post-last injection) and long days (at pre-injection and 1 day post-last injection) to investigate possible time*treatment, time, and treatment interactions. Time denotes repeated measures for blood sampling pre and post treatment, and there are two post treatment samples for short days. Significant time*treatment interaction results were explored by determining (a) whether the trait changed with time within individual treatment groups though Repeated Measure ANOVA GLMs, and (b) using paired t-tests to investigate the direction of these changes, and (c) if at each of the sampling times there were differences in the trait by treatment using standard GLMs. Significant time results were explored through paired t-tests. Additionally, we performed standard GLMs to determine if plasma E2, VTG, oviduct and ovary mass were affected by treatment at 1 day post-injection on long days, and we examined LS means to assess significant differences by treatment. Furthermore, we coded treatment group combinations from short days and long days and used standard GLMs to investigate whether E2 treatment on short days affected plasma E2 or oviduct mass at 1 day post-injection on long days. We also ran Pearson Correlations for mass, hematocrit, plasma

leptin-like immunoreactivity and plasma triglyceride at all sampling periods during short days and long days, examined possible correlations between changes in mass and changes in plasma leptin-like immunoreactivity (including data from capture), and we tested for correlations between both plasma E2 and oviduct mass with plasma VTG, leptin-like immunoreactivity, and triglyceride 1 day post-injection on long days. Finally, Repeat Measure ANOVA GLMs were used to assess possible changes in plasma leptin-like immunoreactivity from transition to long days. All means report actual values, and the figures report non-transformed data for simplicity. Additionally, means are reported in Tables 3.2 and 3.3 for convenience (short day and long day experiments respectively). Means are reported ± standard error. P-values for all multiple comparisons of LS means were Tukey-Kramer adjusted.

3.4 Results

There were no differences in initial body mass, plasma triglyceride, plasma leptin, or hematocrit prior to the SD experiment in females assigned to different treatments ($P > 0.43$ in all cases), i.e. females were randomly assigned to treatments.

3.4.1 Effects of Short Day Photoperiod and Estradiol Treatment

There was a significant time*treatment interaction ($F_{4,34} = 8.28$, $P < 0.0001$) for body mass on SD. Body mass significantly decreased with time for each treatment ($P < 0.004$ in all cases). Vehicle-injected females showed a greater decrease in mass between pre-injection and 1 day post-last injection (-4.61 ± 0.65 g; $t = -7.10$, $P = 0.0009$) compared with non-manipulated (-3.38 ± 0.45 g; $t = -7.56$, $P = 0.0003$) or E2-treated (-1.86 ± 0.48 g; $t = -3.89$, $P = 0.006$) females. Similarly, vehicle-injected females showed a

greater decrease in mass between pre-injection and 7 days post-last injection (-4.67 ± 0.54 g; t = -8.61, P = 0.0003) compared with non-manipulated (-1.68 ± 0.27 g; t = -6.13, P = 0.0009) or E2-treated (-1.60 ± 0.37 g; t = -4.36 P = 0.0033) females (Fig. 3.2a). However body mass was not different among treatments for any of the three sample periods (pre-injection or days 1 and 7 post-injection, $P > 0.49$ in all cases), and the other traits we measured were independent of mass (see below) so we did not control for body mass in subsequent analyses.

Plasma leptin-like immunoreactivity was independent of time ($F_{2,16} = 0.71$, $P >$ 0.50) and treatment ($F_{2,17} = 0.03$, $P > 0.97$) and showed no time*treatment interaction ($F_{4,32} = 0.71$, $P > 0.59$; Fig. 3.3c). There was no correlation between change in mass and change in plasma leptin-like immunoreactivity from capture to pre-injection ($r = 0.066$, $n = 20$, $P > 0.78$), or between pre-injection and either day 1 or day 7 post-injection ($P >$ 0.47 in both cases, Fig. 3.4a,b).

Plasma triglyceride levels were independent of treatment ($F_{2,17} = 1.35$, $P > 0.28$) and the time*treatment interaction was not significant ($F_{4,32} = 0.21$, $P > 0.92$); however, there was a significant time effect ($F_{2,16} = 4.00$, $P < 0.04$). Plasma triglyceride levels decreased from pre-injection to day 1 post injection (-0.30 ± 0.10 mg/mL; t = -3.09, n = 20, P = 0.0061; Fig. 3.3a), but were not different between pre-injection and day 7 post injection ($P > 0.24$; data pooled for all treatment groups). There were no significant correlations between change in mass and change in triglyceride for any time interval (P > 0.38 in both cases). Plasma triglyceride levels were low (pre-Injection: 1.40 ± 0.08 mg/mL; day 1 post, 1.10 ± 0.07 mg/mL; day 7 post, 1.32 ± 0.08 mg/mL), and not within the expected range for yolk precursors (VLDLy; see Discussion). Plasma VTG was only

analyzed in E2-treated females on day 1 post injection for the SD experiment (for comparison with LD data) and was also low (0.12 ± 0.26 μg/mL; not significantly different from zero, $P > 0.65$).

Hematocrit was independent of treatment ($F_{2,17} = 0.68$, $P > 0.51$) and there was no time*treatment interaction ($F_{4,32} = 0.63$, $P > 0.64$); however, there was a significant time effect ($F_{2,16}=5.45$, $P < 0.016$). Hematocrit increased significantly (+1.44 ± 0.61 %) over all groups from 47.2 ± 0.5 % pre-injection to 48.8 ± 0.6 % on day 7 post injection ($t = 2.35$, $n = 20$, $P < 0.03$; Fig 3.2c).

We tested for correlations between plasma leptin-like immunoreactivity, plasma triglyceride, hematocrit and body mass, for each sampling period separately. At the Bonferonni-corrected P value (P = 0.0083) there were no significant correlations.

3.4.2 Effects of Long Day Photoperiod and Estradiol Treatment

3.4.2.1 Plasma Estradiol

E2 injections successfully elevated plasma E2 ($F_{2,18} = 7.46$, $P = 0.0044$) in the LD experiment; females receiving 100 μl (2 μg/g) E2 had higher plasma E2 levels (623.6 ± 86.8 pg/mL) than females receiving vehicle (250.7 ± 86.8 pg/mL, $P = 0.0032$); females receiving 50 μl (1 μg/g) E2 had intermediate values (429.4 ± 86.8 pg/mL). Plasma E2 was highly positively correlated with VTG 1-day post last injection ($r = 0.616$, n = 19, $P = 0.005$). Plasma E2 levels in the LD experiment were not different between individuals previously treated with E2 or vehicle only during the SD experiment for either 100 μl females ($P > 0.99$) or 50 μl females ($P > 0.98$), suggesting no 'priming' effect of E2 on the efficiency of E2 to elevate plasma levels on LDs (though the sample size was small).

Plasma E2 was not correlated with plasma triglyceride or plasma leptin-like immunoreactivity 1-day post last injection ($P > 0.57$ in both cases).

3.4.2.2 Non-Reproductive Traits

There was a significant time*treatment interaction for body mass on LDs ($F_{2,18} = 3.84$, $P = 0.041$): body mass did not change with time for either of the E2-treated groups ($P > 0.29$ in both cases) but decreased with time in the vehicle-treated females (-1.92 ± 0.50 g; $t = -3.81$, $P = 0.0088$, Fig 3.2b.). There were no significant correlations between body mass, hematocrit, plasma triglyceride or plasma leptin-like immunoreactivity for either sampling period (Bonferroni-adjusted $P > 0.0083$ in all cases); therefore, we did not control for body mass in subsequent analyses.

There was a significant time*treatment interaction for plasma leptin-like immunoreactivity ($F_{2,18} = 3.62$, $P < 0.05$), but this was solely due to the vehicle-treated females: plasma leptin-like immunoreactivity was only significantly different among treatments at pre-injection ($P < 0.032$), and varied with time only in the vehicle group (-6.24 ± 1.75 ng/mL; $t = -3.56$ $P < 0.012$; Fig. 3.3d). In addition, there was no correlation between change in mass and change in plasma leptin-like immunoreactivity from pre-injection to day 1 post injection ($r = 0.417$, $n = 21$, $P < 0.06$; this trend was driven by one outlier with a 7.53 g change in mass (i.e. > 2 standard deviations from the mean (-0.36 g). When this bird was removed from analysis, there was no correlation between change in mass and change in plasma leptin-like immunoreactivity ($r = 0.332$, $n = 20$, $P > 0.15$; Fig. 3.4c).

Hematocrit was independent of treatment ($F_{2,18}=0.89$, $P > 0.42$) and the time*treatment interaction ($F_{2,18} = 2.50$, $P > 0.10$), but there was a significant time effect

($F_{1,18}$=11.71, P=0.003). Hematocrit significantly increased (+2.3 %) with time over all treatment groups pooled: 47.8 ± 0.7 % pre-injection, to 50.0 ± 0.7 % day 1 post injection (t = 3.19, n = 21, P < 0.005; Fig. 3.2d).

3.4.2.3 Reproductive Traits

Plasma triglyceride levels were independent of time ($F_{1,18}$ = 0.97, P > 0.33), treatment ($F_{2,18}$ = 0.91, P > 0.42), and had no time*treatment interaction ($F_{2,18}$ = 1.40, P > 0.27; Fig 3.3b). Plasma triglyceride levels were low (pre-Injection: 1.42 ± 0.09, day 1 post-injection: 1.31 ± 0.13) and not within the expected range for breeding values for VLDLy yolk precursors (see Discussion). In contrast, plasma VTG showed a highly significant and dose-dependent treatment effect ($F_{2,16}$ = 66.32, P < 0.0001; Fig. 3.5). Plasma VTG levels were higher in females receiving 100 µl of E2 solution (2.16 ± 0.29 µg/mL) than in females receiving 50 µl of E2 solution (0.79 ± 0.31 µg/mL; P = 0.0152) and vehicle-treated females (0.09 ± 0.31 µg/mL; P < 0.0001). Plasma VTG levels were also significantly higher in the 50 µl E2 females than the vehicle females (P < 0.0001), and levels in vehicle females were not significantly different from zero (P > 0.77).

Ovary wet mass did not vary with treatment ($F_{2,17}$ = 0.51, P > 0.60; Fig. 3.6b): all females had fully regressed ovaries with no evidence of yolky follicle development on day 12 of LD photostimulation. In contrast, oviduct dry mass did vary with treatment ($F_{2,18}$ = 44.66, P < 0.0001). Females receiving 100 µl E2 solution had higher oviduct mass (0.084 ± 0.012 g) than females receiving 50 µl E2 solution (0.031 ± 0.012 g, P = 0.0041). Vehicle-treated females had lower oviduct mass (0.008 ± 0.012 g) than both E2-treated groups (P < 0.0001 in both cases; Fig. 3.6c). Oviduct dry mass was positively

correlated with plasma VTG levels ($r = 0.855$, $n = 19$, $P < 0.0001$) but not with plasma triglyceride or leptin-like immunoreactivity post-injection ($P > 0.10$).

There was a significant effect of SD*LD treatment on dry oviduct mass ($F_{4,16} = 52.63$, $P < 0.0001$), i.e. there was evidence of a 'priming' effect of SD E2 treatment on the response to E2 on long days. In females receiving 100 µl E2 solution, oviduct mass was significantly greater in females previously treated with E2 on SDs (0.116 ± 0.010 g) compared with females treated with vehicle only on SD (0.040 ± 0.012 g, $P = 0.0043$ Fig. 3.7a,b).

3.4.3 Effects of Shift to Long Day Photoperiod

Plasma leptin-like immunoreactivity was unaffected by treatment during the SD experiment; therefore, we pooled treatment groups to analyze changes in plasma leptin-like immunoreactivity for individuals between 7 days post-injection on SDs and pre-injection on LDs to determine if the photoperiodic change to LDs affected plasma leptin-like immunoreactivity. There was no significant change in plasma leptin-like immunoreactivity during the transfer from SDs to LDs ($F_{1,26} = 0.60$, $P > 0.44$). In addition, because plasma leptin-like immunoreactivity was significantly greater in the vehicle group at pre-treatment on LDs compared to the E2 treatment groups and significantly decreased at 1 day post injection, we also tested whether plasma leptin-like immunoreactivity was significantly different between 7 days post-injection on SDs and both pre-injection and 1 day post-injection on LDs in this group, but neither change was significant (respectively, $F_{1,8} = 1.86$, $P > 0.20$; $F_{1,8} = 2.10$, $P > 0.18$).

3.5 Discussion

3.5.1 Overview

We exposed female European starlings to non-stimulatory short (8L:16D) and stimulatory long (18L:6D) day lengths coupled with exogenous estradiol treatment to examine whether plasma leptin-like immunoreactivity responds to estrogen and/or photoperiod (increased day length). Secondarily, we were interested in the effects of long-day photostimulation and exogenous estrogen on activation of the hypothalmo-pituitary-gonadal axis downstream of the pituitary: ovarian steroid (estrogen) secretion and folliculogenesis, vitellogenesis or yolk precursor production, and oviduct growth. We confirmed that birds perceived the transfer to long days as photostimulatory, because this transfer resulted in beak color changes from black to yellow, typical of the onset of gonadal recrudescence (Witschi and Miller 1938; data not shown). In addition, E2 treatment successfully increased plasma E2 concentrations (within physiological ranges) in a dose-dependent manner during LD photostimulation. However, the main result of this study was that, contrary to our predictions, plasma leptin-like immunoreactivity was unaffected by either photoperiod or estrogen treatment. We also found little evidence for consistent relationships between plasma leptin-like immunoreactivity and either body mass or plasma triglyceride and thus, it is unlikely that possible treatment effects were obscured by interactions between plasma leptin-like immunoreactivity and these indices. In contrast, exogenous E2 treatment on 18L:6D did stimulate an increase in plasma levels of one of the two main yolk precursors, plasma VTG (although VLDL remained at basal concentrations). Oviduct growth was also stimulated—though not to levels typical of

breeding birds—but ovary and yolky follicle development were not stimulated. LDs alone had no stimulatory effect on plasma VTG or VLDL.

3.5.2 Lack of Treatment Effects on Plasma Leptin-Like Immunoreactivity

We found no effect of either photoperiod or exogenous E2 treatment on plasma leptin-like immunoreactivity in captive female European starlings. These results contrast with several previous studies in mammals (e.g. Siberian or Djungarian hamsters and Soay rams) which have reported that serum leptin levels do vary with changes in photoperiod (see Introduction), though similar data are not available for other birds. For example, Horton et al (2000) demonstrated that Siberian hamsters on short day length photoperiods (6L:18D) had lower serum leptin concentrations than on long day length photoperiods (16L:8D), as well as lower body weights. While we did find that plasma leptin-like immunoreactivity decreased between pre-injection and 1 day post-injection on LDs among the group of females that remained un-injected with E2 during both the SD and LD experiments, this appears to be an artifact of increased plasma leptin-like immunoreactivity in this group at pre-injection on LDs; the change in this group between 7 days post-injection on SDs and 1 day post-injection on LDs was not significant. Some studies further suggest that changes in plasma leptin levels in response to photoperiod might be modulated by reproductive 'readiness' (Drazen et al. 2000), although other studies contradict this (Atcha et al. 2000). Drazen et al. (2000) found that Siberian hamsters on short day lengths with functional gonads had high serum leptin levels, similar to hamsters on long day lengths with functional gonads; and they concluded that leptin concentrations reflected the short day length photoperiodic reproductive responsiveness of the individual. In contrast, Atcha et al. (2000) found that

administration of leptin in hamsters on either long or short day lengths did not alter pituitary LH concentrations, testosterone concentrations or testicular size, and they concluded that there was no evidence to support the idea that photoperiod induced changes in reproductive function are mediated by plasma leptin levels.

Although we found no effect of E2 treatment on plasma leptin-like immunoreactivity, several studies have reported that leptin plays a role in reproduction in mammals and birds, including regulation of ovarian function and gonadal steroid production (see Introduction). For example, fasting-induced negative reproductive effects in hens, including decreases in LH, progesterone, and estradiol, as well as follicular regression and apoptosis and cessation of egg-laying, were attenuated by injections of purported chicken recombinant leptin (Paczoska-Eliasiewicz et al. 2003). Given that reproduction, body mass, feeding and adiposity signalling can be tightly linked (reviewed in Chehab 2000), we also examined possible relationships between plasma leptin-like immunoreactivity and metabolic indices. However, we found little evidence for systematic relationships between plasma leptin-like immunoreactivity, plasma triglycerides and body mass, even in LD-photostimulated and E2-treated females, which clearly showed evidence of onset of reproductive activity. Previous studies have reported a strong relationship between plasma leptin and mass in birds and mammals (Halaas et al. 1995, Considine et al. 1996, Lohmus et al. 2006, Shi et al. 2006). However, the range of body masses, and presumably, the range of variation in adiposity in these studies was likely much greater than in our study (See Chapter 2); for example, the range of percent body fat in one human study was between approximately 5-65 % (Considine et al. 1996). The lack of any relationship between plasma triglyceride and leptin-like

immunoreactivity in our study also contrasts with Lohmus et al.'s (2006) study where Japanese quail administered with purported chicken leptin for two weeks through osmotic pumps tended to have lower plasma triglyceride levels than control quail. Our study suggests that neither increased day length nor elevated E2 concentrations associated with the onset of reproductive activity causes increases in plasma leptin-like immunoreactivity in captive female European starlings. However, it is important to note that even with LD (16L:8D) photostimulation and exogenous E2 treatment (100 μL), mean yolk precursor levels were only 6.6% and 54.8% for plasma VLDL and VTG respectively, oviduct wet mass was only 11.3% of levels observed in free-living breeding starlings at the 1-egg stage (Christians and Williams 1999b, Vezina and Williams 2003), and none of the females developed yolky follicles; thus, these treatments did not stimulate full reproductive development (see below). Therefore, it remains possible that elevated plasma leptin-like immunoreactivity is related to later stages of reproductive development, such as folliculogenesis and rapid yolk development, or perhaps elevated plasma leptin-like immunoreactivity levels even spur the increases in estradiol associated with egg formation.

3.5.3 Effects of Photostimulation and Estrogen on the Reproductive Axis

Day length is the main proximate factor regulating cycles of gonadal maturation and regression, i.e. seasonal reproduction, in temperate-zone birds (reviewed in Dawson 2008), and it is well documented that exposure to artificial long day lengths in captive birds is sufficient to "switch on" the hypothalamic-pituitary-gonadal (HPG) axis and initiate gonadal recrudescence (Dawson et al. 1985, Nicholls et al. 1988). In male birds, long day length photostimulation alone is sufficient to stimulate gonadal androgen

secretion and spermatogenesis, i.e. reproductive events "downstream" of the pituitary. However, Dawson and Goldsmith (1983) showed that in female European starlings, pituitary gonadotropins (plasma LH and FSH levels) were elevated two weeks after transfer from 8L:16D to 18L:6D; thus, females are clearly photoresponsive at the level of the pituitary. However, in contrast to onset of LD-dependent steroidogenesis and spermatogenesis in males, captive female birds rarely initiate folliculogenesis, develop yolky follicles, or lay eggs, under long day lengths (Farner et al. 1966), although few studies appear to have measured these reproductive traits in females in standard photoperiodic experiments. Our results suggest that long day photostimulation alone is insufficient to stimulate full reproductive development at the level of the oviduct, ovary (steroidogenesis and folliculogenesis), and liver (vitellogenesis). Female starlings exposed to 18L:6D, but not E2, had fully regressed oviducts and baseline levels of both yolk precursors, VTG and VLDLy. We also suggest that these females had baseline ovarian estrogen secretion since any elevation in plasma E2 would have been reflected in elevated VTG levels (Williams and Martyniuk 2000).

Ovarian and oviduct function, and vitellogenesis (increase in plasma yolk precursors, which are yolk targeted very low density lipoprotein, VLDLy, and VTG, both produced by the liver) are known to be E2-dependent, and in seasonally-breeding birds, onset of reproduction is associated with an increase in endogenous E2 (Schjeide et al. 1963, Wallace 1985, Walzem 1996, Walzem et al. 1999, Williams 1999a, Johnson 2000). Treatment of non-breeding females and even immature birds (males or females) with exogenous E2 also increases plasma yolk precursor levels and oviduct growth in a dose-dependent manner (Schjeide and Lai 1970, Yu and Marquard 1973a, 1973b,

Bergink et al. 1974, Deeley et al. 1975, Chan et al. 1976, Lien et al. 1985, Christians and Williams 1999a, Williams 1999b). However, elevating plasma E2 by manipulation fails to initiate later stages of reproductive development: folliculogenesis and ovary growth (Breneman 1956, Lien et al. 1985, Christians and Williams 1999a, reviewed in Williams 1999a).

Our results are in part consistent with findings in previous studies (see above), but the response to exogenous E2 in our study was photoperiod-dependent, and E2 treatment resulted in an elevation in VTG not VLDLy (as measured by plasma triglyceride). In 100 μL E2-treated (2 μg/g) females on 18L:6D, mean plasma VTG concentrations (2.16 ± 0.29 μg/mL) were slightly higher than mean values reported in wild female European starlings with a single yolky follicle (1.66 ± 0.33 μg/mL), but only half the mean value reported for plasma VTG in females with a full follicle hierarchy (4.01 ± 0.23 μg/mL; Challenger et al., 2001). However, plasma triglyceride levels were not affected by E2 treatment on either SDs or LDs, i.e. triglyceride concentrations were too low to be in the range of yolk precursors (VLDLy) in both experiments. Mean plasma triglyceride concentrations after E2 treatment on both SDs and LDs (<1.60 ± 0.23 mg/mL in all cases) were well below levels reported by Challenger et al. (2001) for females with a single yolky follicle (10.7 ± 2.4 mg/mL) and females with a full follicle hierarchy (19.4 ± 1.4 mg/mL), and even below levels in non-breeding females (3.5 ± 0.4 mg/mL). Plasma VTG levels, but not triglyceride levels, were also strongly positively correlated to plasma E2 levels. This result differs from that in captive non-breeding female zebra finches where E2 treatment resulted in an increase in both plasma VLDL and VTG comparable to concentrations found in breeding females (Williams 1999b). However, female zebra

finches also readily lay eggs and rear chicks in captivity even on constant LD photoperiods (14L:10D; Williams 1996). Christians and Williams (1999a) have shown that manipulation of plasma E2 (using silastic implants) in free-living European Starlings resulted in higher plasma VTG concentrations at the 1-egg stage and higher plasma VTG and VLDL concentrations and greater oviduct mass at clutch completion relative to control birds. Thus, our results suggest that the combination of E2-treatment and LD photostimulation was necessary to elevate plasma VTG in female starlings, but that some other factor(s) is required to stimulate other components of the reproductive system (see Dawson 2008 regarding plasticity in gonadal maturation).

Our data on changes in reproductive organs supports this interpretation. Among females on 18L:6D with no E2 treatment, wet oviduct mass was 0.038 g, which is less than in non-breeding female European starlings (0.084 g; T.D. Williams unpubl. data), suggesting that oviducts were fully regressed despite LD photostimulation. In 100 μL E2-treated (2 μg/g) females on 18L:6D, mean wet oviduct mass was 0.451 g, which is approximately 11% of mean oviduct mass in laying females (4 g; Vezina and Williams 2003). This finding is consistent with a study on non-breeding Zebra finches, wherein exogenous E2 increased oviduct mass in treated females four times more than oviduct mass in control birds, and yet the oviducts in treated females still only reached 30% of mature oviduct mass (Williams 1999b). Additionally, in our study, no females (regardless of treatment) initiated development of yolky follicles, and ovary mass did not vary with photoperiod or E2 treatment. Our results were similar to findings from a study in which non-photostimulated Bobwhite quail *(Colinus virginianus)* that were injected with β-estradiol-3-benzoate had a dose dependent linear increase in oviduct mass without

a corresponding increase in ovary mass (Lien et al. 1985). We suggest that the incomplete response of the reproductive axis was caused by the necessity for female European starlings (unlike males) to experience additional environmental and/or social cues to initiate full reproductive development and, specifically, onset of vitellogenesis, yolk uptake, follicle development and egg-laying. This might be due to the fact that these specific later stages of reproductive maturation are particularly costly to the female. For example, there is evidence that the oviduct is an energetically-expensive organ to maintain (Vezina and Williams 2003, Williams and Ames 2004), and changes in lipoprotein metabolism associated with synthesis of yolk targeted VLDL (VLDLy) might decrease the availability of 'generic' VLDL which the female needs to meet her own metabolic needs (Salvante et al. 2007).

Finally, we found evidence of a 'priming effect' of E2 on sensitivity or response to subsequent E2 treatment. When exposed to E2 on LDs, females treated with E2 during the SD experiment had larger oviducts than females that were vehicle injected during SDs. There are several studies documenting priming effects of estrogens in birds; for example Bergink and Wallace (1974) described a "memory" effect of E2 on VTG synthesis in rooster livers; they showed that VTG synthesis was more rapid after the second dose of E2 than the first dose, even if the second dose was 50 days after the first. In addition, Sockman et al. (2004) demonstrated that among free-living juvenile European starlings placed in captivity, some components of reproductive development induced by photoperiod were enhanced through previous photostimulatory experience, which may support the theory that prior photoperiodic "priming" of the HPG axis could be a factor in age-dependent reproductive success in temperate zone birds. Perhaps, the

priming of the estrogen response during a female's first reproductive season on subsequent increases in estrogen during successive reproductive seasons could offer another possible mechanism underlying age-dependent reproductive success among many birds.

3.5.4 Conclusion

In summary, although we did not find plasma leptin-like immunoreactivity to be responsive to photoperiod or estrogen, it is possible that later stages of reproductive development (i.e. folliculogenesis and rapid yolk development) could elevate plasma leptin-like immunoreactivity; however, the current study only resulted in incomplete stimulation of the reproductive axis even under a long day photoperiod, likely because female starlings require additional environmental or social cues to initiate energetically costly later stages of reproductive development. Indeed, our previous research in free-living female European starlings may support a connection between plasma leptin-like immunoreactivity and reproductive development; we demonstrated that plasma leptin-like immunoreactivity is elevated during egg-formation relative to later reproductive stages (see Chapter 2), similar to plasma E2 concentrations in birds (Dawson 1983, Sockman and Schwabl 1999).

Table 3.1 **Trait means and std. errors for Vehicle 50 and Vehicle 100 groups in the Long Day Photoperiod and Estradiol Treatment Experiment at pre-treatment and 1 day post-injection among captive female European starlings (*Sturnus vulgaris*).**

Trait	Group	Pre-Treatment	Post-Treatment D1
Mass (g)	Vehicle 50	74.85 ± 2.80	72.53 ± 2.69
	Vehicle 100	70.01 ± 2.42	68.39 ± 2.33
Hematocrit (%)	Vehicle 50	46.03 ± 1.62	51.14 ± 2.02
	Vehicle 100	45.48 ± 1.40	49.36 ± 1.75
Triglycerides (mg/mL)	Vehicle 50	1.60 ± 0.24	1.49 ± 0.35
	Vehicle 100	1.35 ± 0.21	1.11 ± 0.30
Leptin (ng/mL)	Vehicle 50	27.83 ± 1.72	20.87 ± 1.77
	Vehicle 100	27.98 ± 1.49	22.28 ± 1.54
Estradiol (ng/mL)	Vehicle 50	-	184.0 ± 134.7
	Vehicle 100	-	300.8 ± 116.7
Vitellogenin (μg/mL)	Vehicle 50	-	0.08 ± 0.56
	Vehicle 100	-	0.10 ± 0.40
Oviduct Wet Mass (g)	Vehicle 50	-	0.0443 ± 0.1088
	Vehicle 100	-	0.0325 ± 0.0942
Oviduct Dry Mass (g)	Vehicle 50	-	0.0086 ± 0.0181
	Vehicle 100	-	0.0077 ± 0.0157
Ovary Wet Mass (g)	Vehicle 50	-	0.0253 ± 0.0052
	Vehicle 100	-	0.0367 ± 0.0045

Table 3.2 **Trait means and std. errors by treatment group in the Short Day Photoperiod and Estradiol Treatment Experiment at pre-treatment and 1 day and 7 days post-injection among captive female European starlings (*Sturnus vulgaris*).**

Trait	Group	Pre-Treatment	Post-Treatment D1	Post-Treatment D7
Mass (g)	Ctl	74.00 ± 1.92	70.62 ± 1.84	72.32 ± 1.85
	Veh	75.76 ± 2.08	71.15 ± 1.99	71.09 ± 1.10
	E2	75.35 ± 1.80	73.49 ± 1.72	73.75 ± 1.73
Hematocrit (%)	Ctl	47.15 ± 1.00	47.30 ± 1.08	49.00 ± 1.16
	Veh	46.32 ± 1.00	45.54 ± 1.08	48.39 ± 1.16
	E2	47.99 ± 0.87	47.76 ± 0.93	48.63 ± 1.01
Triglycerides (mg/mL)	Ctl	1.54 ± 0.14	1.16 ± 0.15	1.48 ± 0.14
	Veh	1.27 ± 0.14	1.10 ± 0.15	1.17 ± 0.14
	E2	1.38 ± 0.12	1.04 ± 0.13	1.26 ± 0.12
Leptin (ng/mL)	Ctl	24.6 ± 1.2	24.6 ± 1.4	24.1 ± 1.5
	Veh	25.4 ± 1.2	23.1 ± 1.4	25.4 ± 1.5
	E2	24.7 ± 1.0	24.5 ± 1.2	24.9 ± 1.3

Table 3.3 **Trait means and std. errors by treatment group in the Long Day Photoperiod and Estradiol Treatment Experiment at pre-treatment and 1 day post-injection among captive female European starlings (*Sturnus vulgaris*).**

Trait	Group	Pre-Treatment	Post-Treatment D1
Mass (g)	Vehicle	72.08 ± 1.86	70.17 ± 1.78
	E2 50	72.16 ± 1.83	71.65 ± 1.76
	E2 100	71.81 ± 1.83	73.15 ± 1.76
Hematocrit (%)	Vehicle	45.72 ± 1.03	50.12 ± 1.30
	E2 50	49.28 ± 1.03	50.41 ± 1.30
	E2 100	48.24 ± 1.03	49.58 ± 1.30
Triglycerides (mg/mL)	Vehicle	1.46 ± 0.16	1.28 ± 0.23
	E2 50	1.35 ± 0.16	1.06 ± 0.23
	E2 100	1.45 ± 0.16	1.60 ± 0.23
Vitellogenin (μg/mL)	Vehicle	-	0.09 ± 0.31
	E2 50	-	0.79 ± 0.31
	E2 100	-	2.16 ± 0.29
Estradiol (pg/mL)	Vehicle	-	250.7 ± 86.8
	E2 50	-	429.4 ± 86.8
	E2 100	-	623.6 ± 86.8
Leptin (ng/mL)	Vehicle	27.9 ± 1.1	21.7 ± 1.1
	E2 50	24.4 ± 1.1	21.1 ± 1.1
	E2 100	23.7 ± 1.1	23.7 ± 1.1

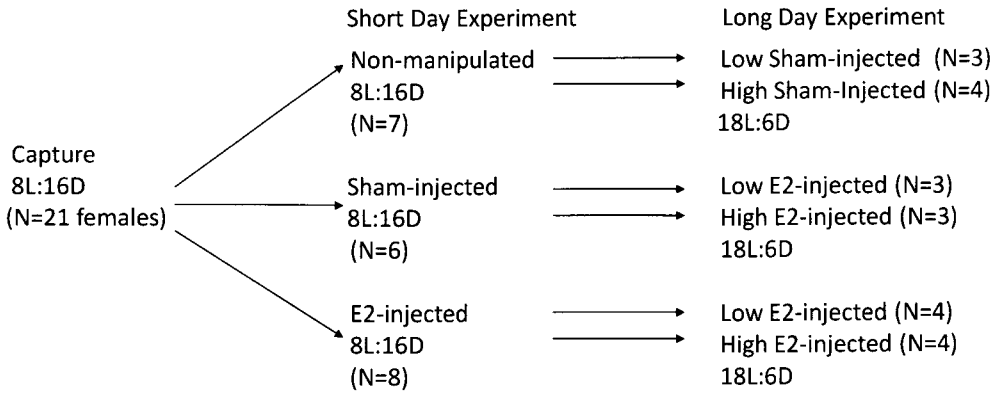

Figure 3.1 **Design of the Short Day and Long Day Photoperiod and Estradiol Treatment Experiments for captive female European starlings (*Sturnus vulgaris*). The SD experiment began 16 full days after capture and commencement of the SD light cycle. The LD experiment began 6 full days after the change to the LD light cycle.**

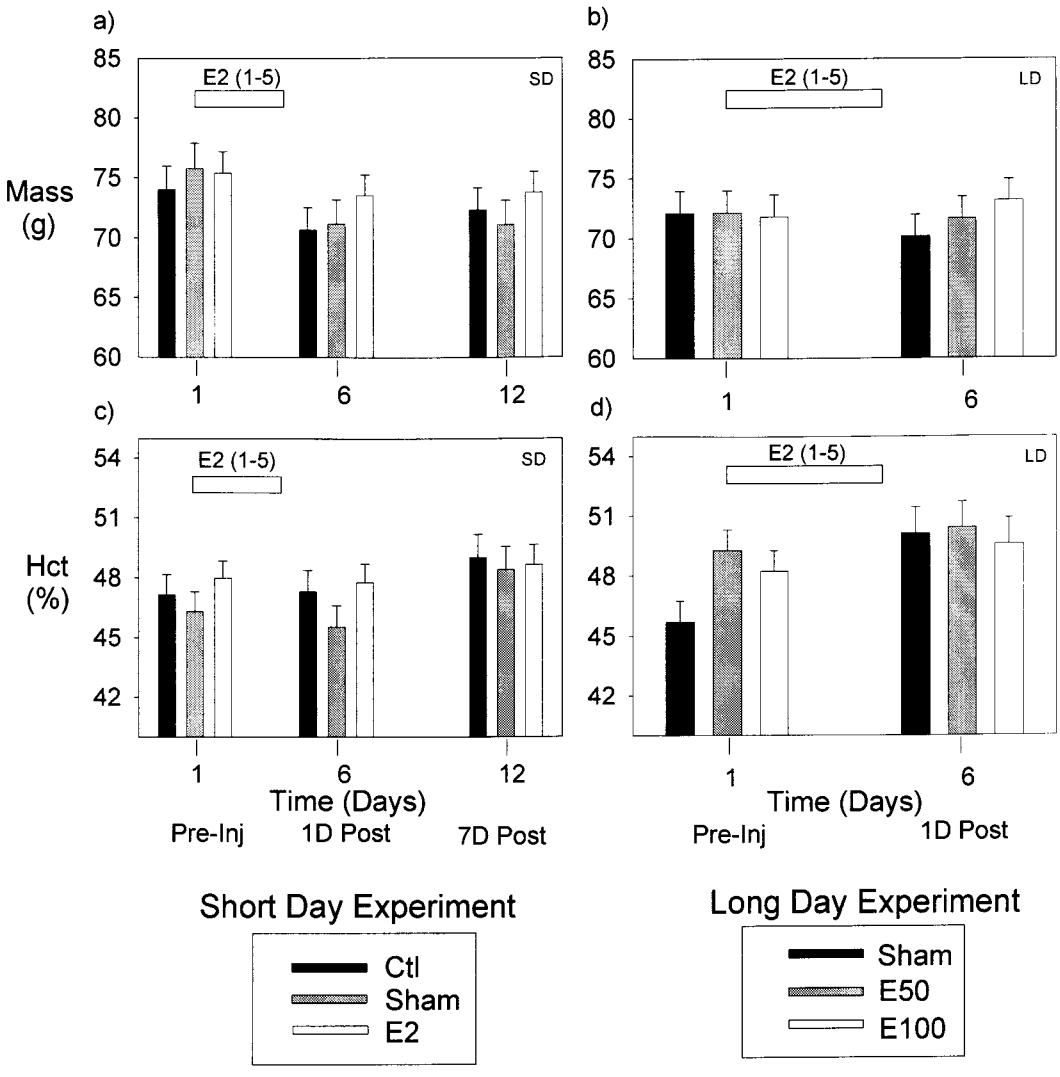

Figure 3.2 **Mass (g) and hematocrit (%) over time during the Short Day and Long Day Photoperiod and Estradiol Treatment Experiments at pre-treatment and 1 and 7 days post-injection during the SD experiment and at pre-treatment and 1 day post-injection during the LD experiment among captive female European starlings (*Sturnus vulgaris)*: (a) mass on Short Days; (b) mass on Long Days; (c) hematocrit on Short Days; and (d) hematocrit on Long Days.**

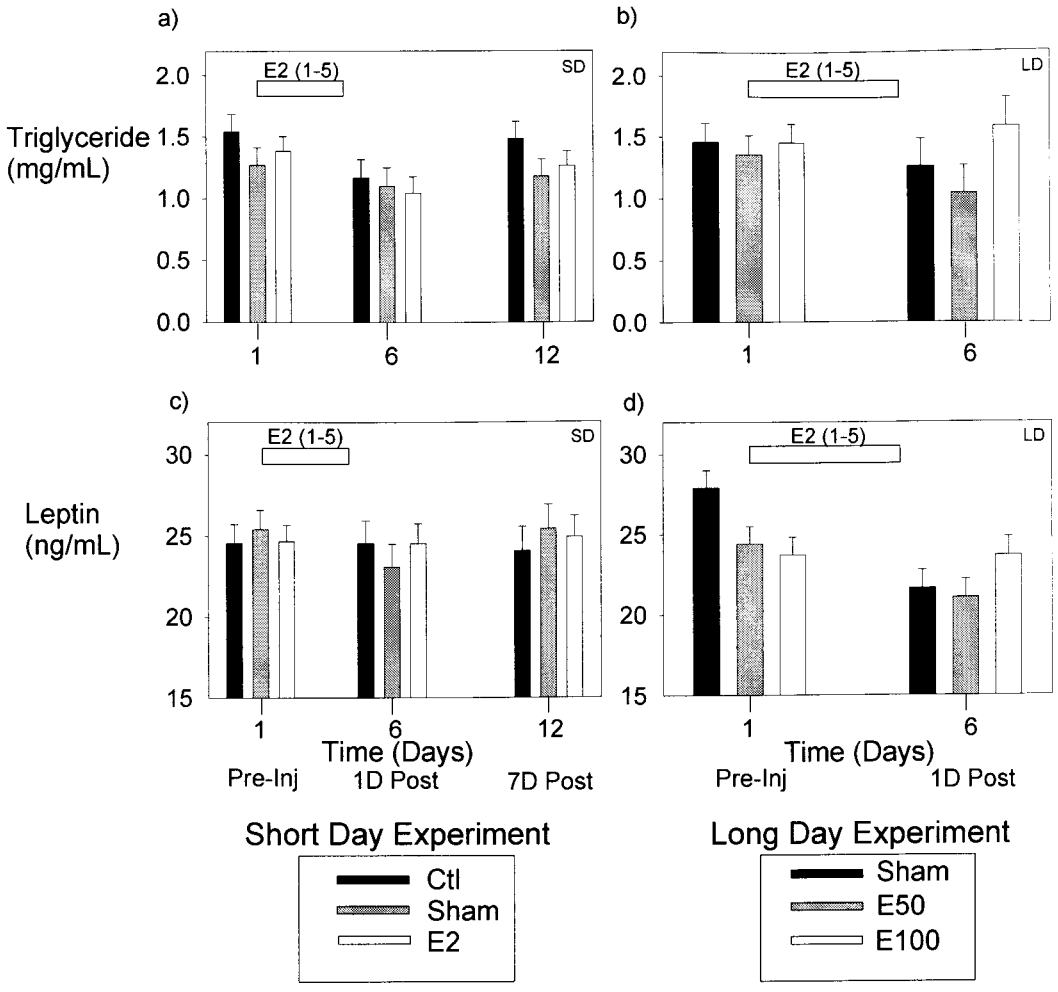

Figure 3.3 **Plasma triglyceride (mg/mL) and leptin-like immunoreactivity levels (ng/mL) over time during the Short Day and Long Day Photoperiod and Estradiol Treatment Experiments at pre-treatment and 1 and 7 days post-injection during the SD experiment and at pre-treatment and 1 day post-injection during the LD experiment among captive female European starlings (*Sturnus vulgaris)*: (a) triglyceride on Short Days; (b) triglyceride on Long Days; (c) leptin-like immunoreactivity on Short Days; and (d) leptin-like immunoreactivity on Long Days.

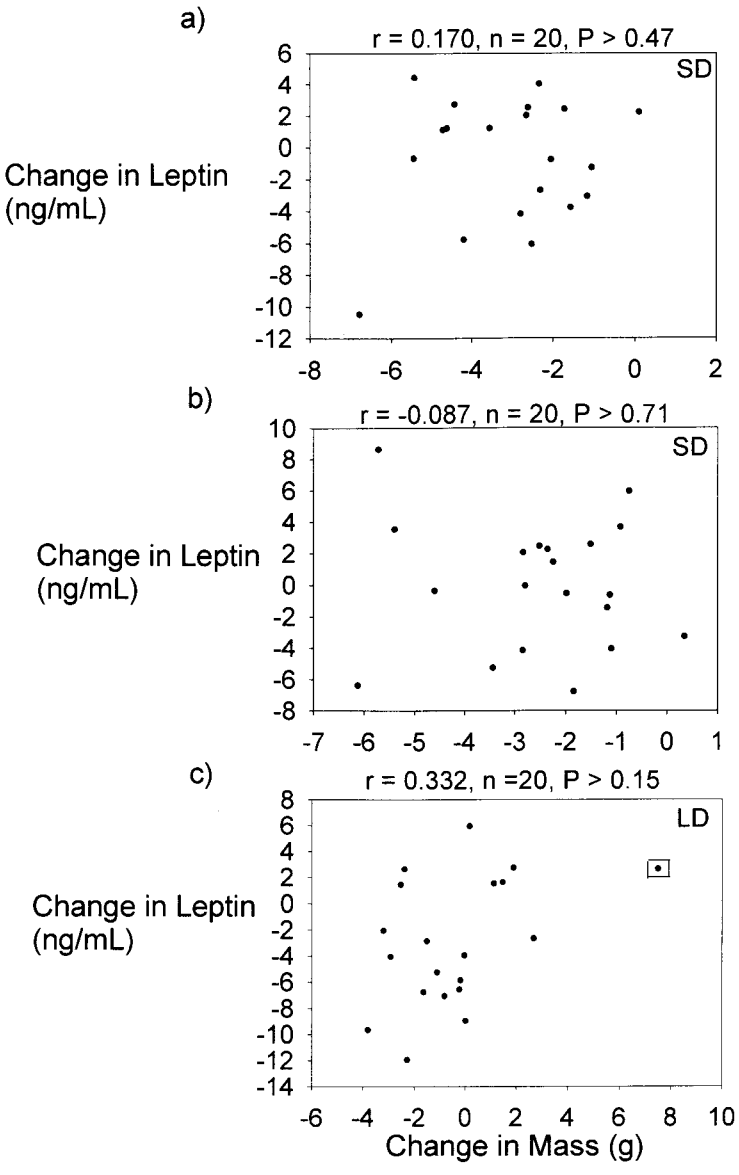

Figure 3.4 **Correlations between change in mass (g) and change in plasma leptin-like immunoreactivity (ng/mL) during SD and LD Photoperiod and Estradiol Treatment Experiments among captive female European starlings (*Sturnus vulgaris*) (a) Short Days 1 Day Post Last Injection – Pre Injection; (b) SD 7 Days Post Last Injection – Pre Injection; (c) LD 1 Day Post Last Injection – Pre Injection (is an outlier, >2 Std. Dev. from the mean, equation excludes this point).**

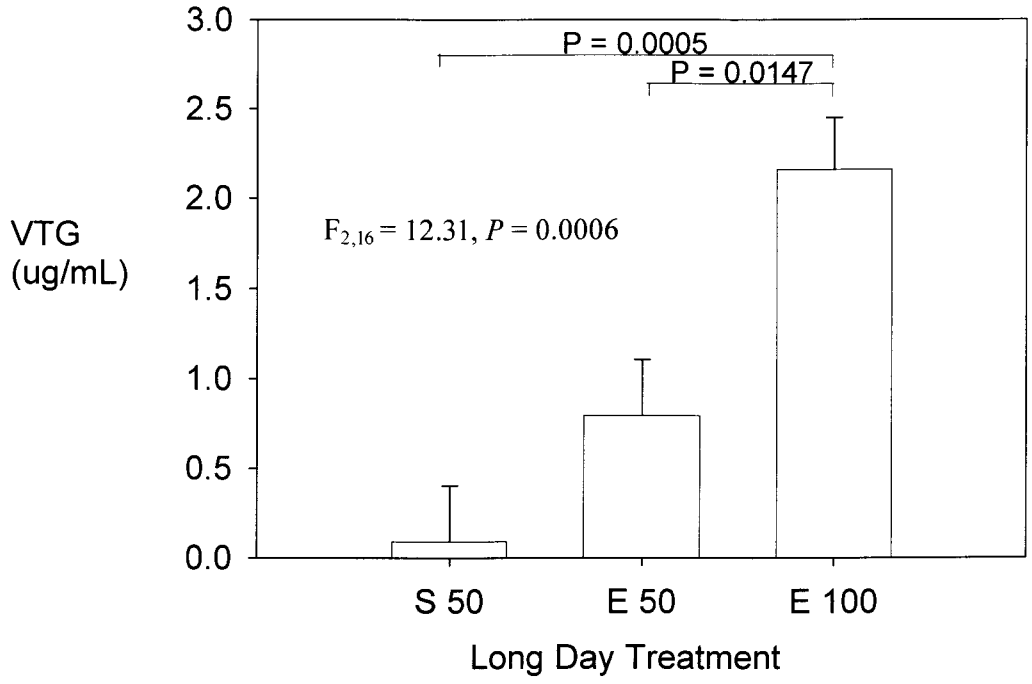

Figure 3.5 **Plasma VTG (ug/mL) 1 day post-last injection during the Long Day Photoperiod and Estradiol Treatment Experiment among captive female European starlings (*Sturnus vulgaris)* injected with either vehicle (S50), 50 µL (E50) or 100 µL (E100) of estradiol. Untransformed data are presented; however, statistics reported in the text for VTG are based on log-transformed data (see Results).**

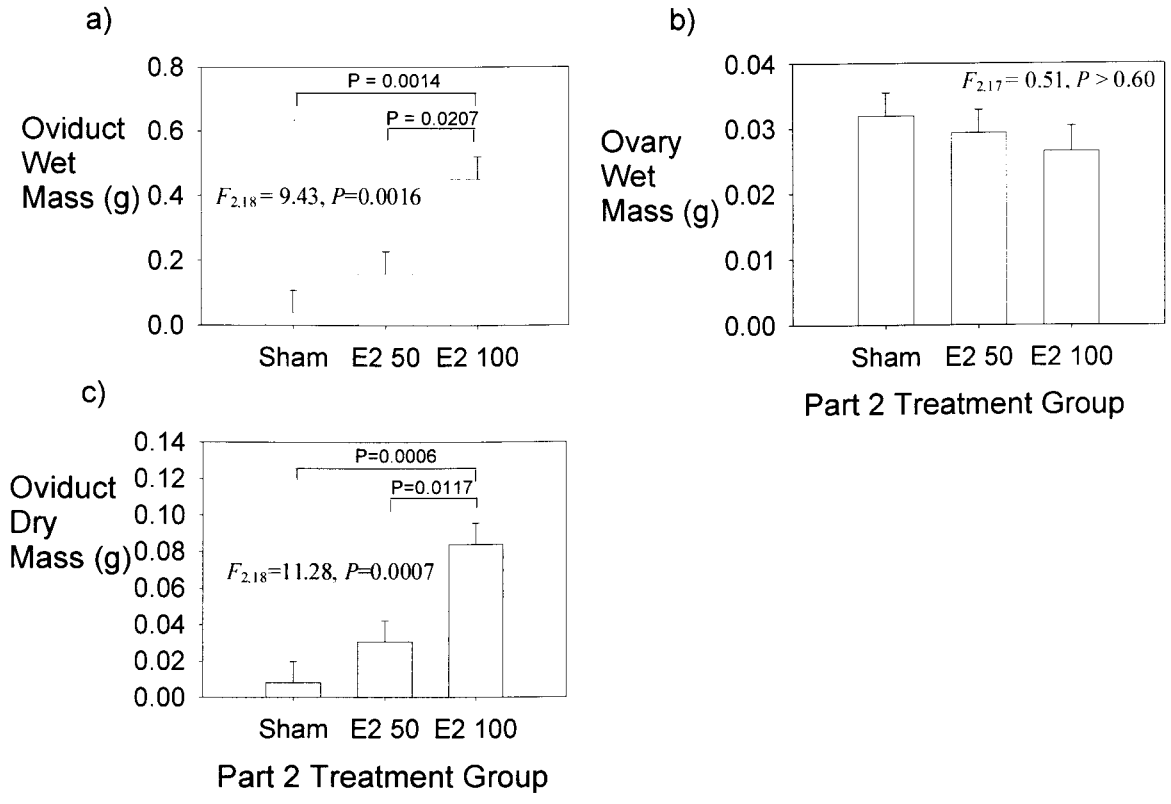

Figure 3.6 **Long Day Photoperiod and Estradiol Treatment Experiment effects among captive female European starlings (*Sturnus vulgaris)* injected with either vehicle (Sham), 50 µL (E2 50) or 100 µL (E2 100) of estradiol on (a) wet oviduct mass (g) ; (b) wet ovary mass (g) ; and (c) dry oviduct mass (g) by treatment group. Untransformed data are presented; however, statistics reported in the text for wet and dry oviduct mass are based on log transformed data (see Results).

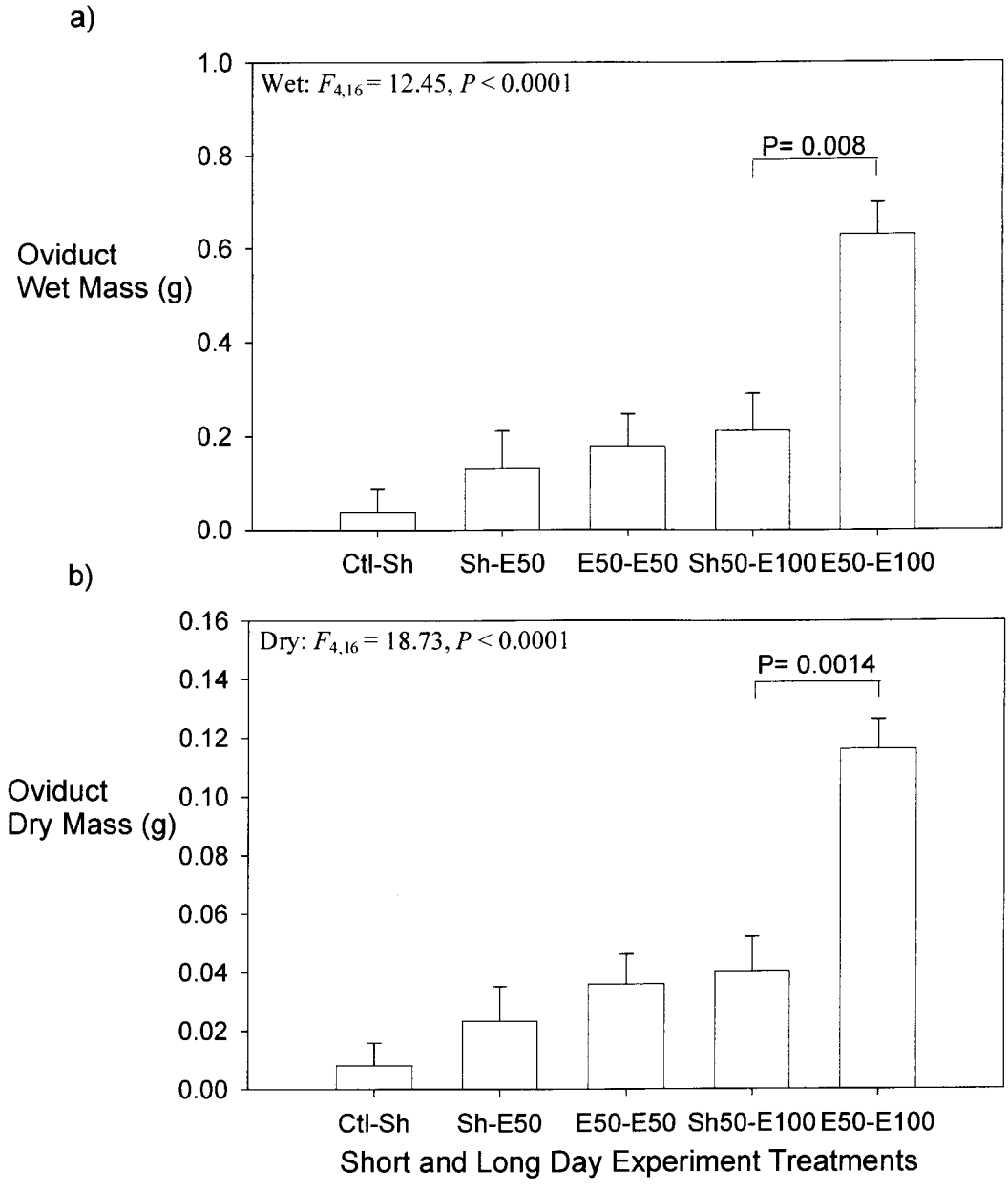

Figure 3.7 **Priming Effects of E2 during the Short Day Photoperiod and Estradiol Treatment Experiment among captive female European starlings (*Sturnus vulgaris*). Females underwent a combination of treatments on SDs and LDs and were either un-injected (ctl), injected with vehicle (Sh), 50 µL of estradiol (E50), or 100 µL (E100) of estradiol. Treatment group combination effects on: (a) wet oviduct mass (g); and (b) dry oviduct mass (g) following the Long Day Photoperiod and Estradiol Treatment Experiment. Untransformed data are presented; however, statistics reported in the text for wet and dry oviduct mass are based on log-transformed data (see Results).**

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Chapter 4 :
CONCLUSIONS

4.1 Overview

The central research objectives for the studies documented in this thesis were to examine patterns of variation in avian plasma leptin-like immunoreactivity among free-living female European starlings (*Sturnus vulgaris*) during the reproductive and non-reproductive seasons, and to investigate possible stimulatory effects of estrogens and photoperiod on plasma leptin-like immunoreactivity in wild-caught females. Plasma leptin-like immunoreactivity significantly varied during the breeding season as well as seasonally among free-living females; circulating leptin-like immunoreactivity was elevated during egg-laying and clutch completion, and decreased at incubation, remaining depressed through chick rearing, but was again elevated in non-breeding (November) birds. In contrast, body mass and body composition significantly decreased between egg-laying and clutch completion, remaining low through incubation and chick rearing, as well as in non-breeding (November) birds. Plasma leptin-like immunoreactivity therefore varied independently from body mass and body composition. Thus, our results suggest that mammalian and captive bird research asserting leptin's strong relationship to body mass and adiposity are not as relevant to wild European Starlings as the relationships we found between plasma leptin-like immunoreactivity and reproductive stage and seasonality (Chapter 2). Next, we investigated some potential causes of elevated plasma leptin-like immunoreactivity during egg-laying using captive females. Specifically, we investigated whether elevated plasma leptin-like immunoreactivity is

stimulated by photoperiodically driven reproductive development and/or the elevated estrogen levels necessary for egg-production. Plasma leptin-like immunoreactivity was unaffected by photoperiod or exogenous estradiol (E2) administration. However, we did observe estrogenic and photoperiodic stimulation of one of the two primary yolk precursors - plasma VTG increased but plasma VLDL did not – as well as a positive dose dependent response in oviduct mass. In contrast, ovary mass and yolky follicle development remained un-stimulated. Therefore, photoperiod and E2 only incompletely stimulated the reproductive axis, so we cannot unequivocally exclude a link between elevated plasma estradiol and plasma leptin-like immunoreactivity during egg-production in free-living birds (Chapter 3).

4.2 Discussion

Our findings that plasma leptin-like immunoreactivity does not vary with body mass and condition contrasts with research in captive birds and mammals purporting strong relationships between circulating leptin and body mass and adiposity. However, many mammalian species as well as captive poultry have greater intra- and inter-individual variation in body mass and adiposity than European starlings; therefore, a correlation between plasma leptin-like immunoreactivity and mass or adiposity would have to be extremely high to be evident in birds whose total percent lipid mass during the reproductive season varies from only 2-4% (Chapter 2). Unfortunately, there is no published research on circulating leptin-like immunoreactivity in free-living birds, thus a comparison between our findings and other studies examining the strength of the relationship between body mass or adiposity and circulating leptin-like immunoreactivity among wild individuals is not possible at this time.

While many studies have demonstrated that leptin is involved in reproduction in both mammals and birds, neither photoperiod nor E2 administration stimulated changes in plasma leptin-like immunoreactivity among our captive starlings. This was somewhat surprising, because leptin and reproductive steroid secretion appear to be linked. For example, leptin administration has been shown to affect the release of several reproductive steroids, including estradiol in birds. However, we conversely tested whether estradiol administration affected leptin-like immunoreactivity. Additionally, although, females in our study were effectively photostimulated by increased day length - their beak color changed typical of the onset of gonadal recrudescence among European starlings - our results do not agree with mammalian studies demonstrating changes in circulating leptin levels due to photoperiod. Unfortunately, there have not been other avian studies examining serum leptin-like immunoreactivity changes with day length manipulations with which to compare our results. However, similar to results from other studies, administration of exogenous E2 in our non-breeding captive females successfully stimulated some, but not all, of the reproductive axis. Namely, similar to other studies, E2 administration increased yolk precursor concentrations – but unlike some other studies, only VTG (not VLDL) increased - and oviduct growth, while E2 administration did not initiate folliculogenesis or ovary growth (also similar to other studies). Our results indicate that an additional cue(s), beyond combined E2 administration and photoperiodic stimulation, is necessary to activate the entire reproductive system (Chapter 3).

4.3 Future Work

The next avenue to investigate a possible relationship between the elevation in plasma leptin-like immunoreactivity and elevated E2 during egg-formation in birds would be to examine plasma leptin-like immunoreactivity and E2 concentrations in conjunction, directly prior to, during, and after egg-formation in free-living birds to ascertain whether they change synchronously. If they do vary synchronously, this is further incentive to pursue a possible relationship between plasma estradiol and plasma leptin-like immunoreactivity, and to investigate the directional causality of this relationship. Firstly, one could design and conduct an experiment wherein captive female birds are stimulated both photoperiodically and by the presence of males (both sexes kept in the same large room at low density) under which *all* aspects of the reproductive axis are stimulated, and either inject E2 or block E2 uptake to examine whether there are E2 treatment effects on plasma leptin-like immunoreactivity. Secondly, another similar experiment should be performed - wherein females are stimulated photoperiodically and by the presence to conspecific males, inducing the response of the entire reproductive axis - in which females are injected with mammalian or putative chicken leptin to determine if exogenous administration of leptin spurs increases in plasma estradiol. If elevations in plasma leptin-like immunoreactivity in captive females during treatment are dependent upon increases in plasma E2, we could then support the hypothesis that elevations in plasma E2 during egg-formation spur increases in plasma leptin-like immunoreactivity in birds; whereas, if administration of exogenous leptin induces elevations in plasma E2, then it is possible that elevated leptin is one of the cues necessary to spur the elevated E2 required for egg formation.

Although, an investigation of possible causes for elevated plasma leptin-like immunoreactivity in starlings in fall (Chapter 2) was not within the scope of this thesis, several experiments could investigate this phenomenon. High plasma leptin-like immunoreactivity in winter could possibly be the result of increased energetic activities, such as foraging or thermogenesis; however, there is no avian research on the effects of cold temperatures on putative leptin. To examine whether increased foraging behavior is at least partially responsible for these elevated levels, a field study could supplement food for one group of birds in a population in fall or winter and leave the other group to forage naturally to determine if leptin-like immunoreactivity increases among supplemented individuals. Food supplementation would likely result in an increase in plasma leptin-like immunoreactivity because body mass (and adiposity) could increase. Foraging behavior is linked to body mass; therefore, it would be difficult to discriminate between plasma leptin-like immunoreactivity effects due to increased foraging activity and effects due to increased body mass and adiposity. To investigate whether elevated plasma leptin-like immunoreactivity levels in winter are in part due to thermogenesis, one could conduct a captivity study wherein some wild-caught individuals would be placed in a warm temperature (same as ambient summer temperature), while others are kept in a cold temperature (same as ambient winter temperature). Additionally, the temperature for the groups could be switched halfway through the experiment (excepting in control individuals who do not undergo a change in temperature), to explore whether plasma leptin-like immunoreactivity levels change. If individuals kept at the warmer temperature have lower leptin immunoreactivity levels than those at the colder temperature, and if birds switched from the colder temperature to the warmer temperature have decreased

leptin-like immunoreactivity levels, and if birds switched from the warmer temperature to the colder temperature have increased leptin-like immunoreactivity levels, then elevated plasma leptin-like immunoreactivity levels in winter could partially be resultant from increased thermogenesis.

4.4 Closing Remarks

The research documented in this thesis is unique because it investigates plasma leptin-like immunoreactivity patterns of variation among both captive and wild birds. All published research on putative avian leptin to date employs captive subjects; however, without the complimentary study of circulating leptin-like immunoreactivity levels among wild individuals, it is not possible to ascertain the ecological validity of putative avian leptin findings. Our findings on the lack of correlation between body mass and condition and plasma leptin-like immunoreactivity in wild starlings contrast with those in captive birds, illustrating just one example of how exceedingly important it is that avian leptin research focuses on both captive and wild individuals. Additionally, while the existence of avian leptin remains controversial because the cDNA sequence in GenBank has not been found in poultry genomes, it is highly likely that avian leptin exists - similar effects have been documented in birds and mammals, and leptin receptor(s) have been identified in poultry (Chapter 1). Regardless of the exact chicken leptin cDNA sequence – which avian leptin researchers hope will soon be discovered/confirmed - avian leptin research should continue to attempt to illuminate the wide-ranging effects of this hormone. Clearly, the interplay of putative leptin in adiposity regulation, feeding behavior, immune function, and reproduction is highly complicated in birds (as in

mammals), and complex field and captivity experiments will be necessary to tease apart the varied and fascinating roles of avian leptin.

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