

Variation in plasma leptin-like immunoreactivity in free-living European starlings (*Sturnus vulgaris*)

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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, the identification and function of leptin in birds remains controversial, and studies have focused on captive or domesticated species. Here, 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), but was elevated again 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, plasma leptin-like immunoreactivity did not parallel changes in body mass or body composition. These data suggest that the strong positive relationship between plasma leptin-like immunoreactivity and body mass reported for captive birds and mammals does not hold for free-living birds. Rather, among free-living female European starlings, variation in plasma leptin-like immunoreactivity is associated with breeding stage or seasonal variation *per se*, and we discuss possible mechanisms underlying this variation, focusing on ovarian function and egg production.

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1. Introduction

Leptin, the protein hormone product of the obese (*ob*) gene secreted by fat cells (adipose tissue), was sequenced and cloned in mice and humans less than 15 years ago (Zhang et al., 1994). Since then, a large amount of research, mainly in mammals, has demonstrated that leptin has diverse physiological roles ranging from regulation of feeding behavior and body weight to effects on reproductive and immune functions (Budak et al., 2006; Friedman and Halaas, 1998; La Cava and Matarese, 2004). Few mammalian studies (excepting human studies) have examined variation in leptin in 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 (Concannon et al., 2001; Hissa et al., 1998; Mustonen et al., 2005; Nieminen et al., 2001, 2002). 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 (Mustonen et al., 2005). In contrast, in woodchucks (*Marmota mon-*

ax), plasma leptin is elevated in late spring and peaks in the summer before decreasing through the winter, following the general trend of body fat gain in spring and summer and loss in fall and winter (Concannon et al., 2001).

Fewer studies have investigated variation in, or the physiological roles of, leptin in birds or other non-mammalian vertebrates. In part, this is likely due to the ongoing controversy regarding the identification of leptin in birds and, specifically, the putative avian leptin gene sequence which has been deposited in GenBank but which has not been identified in the chicken genome to date (Friedman-Einat et al., 1999; Sharp et al., 2008). However, a chicken leptin receptor gene (CLEPR or chLEPR or cOB-R) has been cloned and the structure of the gene resembles mammalian leptin receptor genes (Horev et al., 2000; Ohkubo et al., 2000). Ohkubo et al. (2000) have shown ovarian and hepatic expression of chicken leptin receptor mRNA, as well as expression in the kidneys, intestines, and brains of hens. Additionally, 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).

Furthermore, several studies in birds have used either mammalian leptin or putative recombinant chicken leptin in manipulation studies, or have measured variation in plasma leptin-like immunoreactivity using heterologous leptin radioimmunoassays (RIAs;

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based on recombinant leptin protein derived from the reported gene sequence). These studies strongly suggest that a leptin-like hormone does exist in birds (Simon et al., 2009) with wide ranging physiological effects similar to those reported in mammals, including involvement in feeding behavior, regulation of body mass, and effects on reproduction and immune function (Denbow et al., 2000; Kuo et al., 2005; Lohmus et al., 2004, 2003, 2006; Sirotkin and Grossmann, 2007; Sirotkin et al., 2007). For example, Lohmus et al. (2006) demonstrated that exogenous recombinant chicken leptin administration decreased 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). 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).

The vast majority of published studies on leptin-like function in birds to date have involved captive birds (excepting Kochan et al., 2006; Quillfeldt et al., 2009), and most studies have involved domesticated species, such as chicken and quail. Therefore, little 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 some studies of captive mammals. Studies in free-living birds may inform the debate, and perhaps help resolve the controversy, about the role of leptin-like hormones in non-mammalian taxa (Friedman-Einat et al., 1999; Scanes, 2008; Taouis et al., 1998). In this paper we describe changes in plasma leptin-like immunoreactivity (terminology following Scanes, 2008) during the reproductive and non-reproductive seasons in free-living female European starlings (*Sturnus vulgaris*). The specific objectives of our paper were (a) to 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; (b) to describe seasonal and breeding-stage-specific variation in plasma leptin-like immunoreactivity; and (c) to compare leptin data to the mass data to test the hypothesis that changes in plasma leptin-like immunoreactivity reflect a predominant effect of variation in body or fat mass (see for example, Considine et al., 1996; Halaas et al., 1995; Lohmus et al., 2006; Shi et al., 2006).

2. Methods

2.1. Field site and sample collection

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 and 15 feet off the ground. During the breeding season, all nest boxes were monitored daily between April 1 and July 4, 2007. 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 (± 0.1 g), 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 blood was centrifuged and plasma was frozen at -20 °C until assay analysis.

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. 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 ($n = 4$), (b) at clutch completion (2 days after their final egg was laid) ($n = 9$), (c) at day 8 of incubation ($n = 9$), and (d) between days 11 and 14 of chick-rearing ($n = 14$). In fall, non-breeding birds were mist-netted on November 10, 2007 between 11:00 and 13:30 PST. We blood sampled most breeding birds within 3 min of capture (during which time there is no significant elevation of plasma corticosterone indicating a 'stress response', (Romero and Reed, 2005). Some clutch completion birds were blood sampled up to 1 h after capture but for these birds plasma leptin-like immunoreactivity was independent of handling time ($P > 0.52$). Similarly, time between capture and blood sampling did not affect body mass, size-corrected body mass (mass/tarsus), or plasma leptin-like immunoreactivity ($P > 0.4$ in all cases) in non-breeding birds. Non-breeding females were initially sexed 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 (as part of another study, $n = 21$). We used additional data from European starlings obtained in the 1999–2001 breeding seasons (Williams and Vézina, unpublished sample sizes: egg-laying $n = 55$; clutch completion $n = 39$; incubation $n = 14$; chick-rearing $n = 52$) for comparison of body mass, non-reproductive mass and fat mass with our 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 typical changes in fat mass (not measured in 2007).

2.2. Plasma leptin-like immunoreactivity RIA

Plasma was analyzed for leptin-like immunoreactivity at the United States Department of Agriculture Laboratories, Beltsville, MD, USA, by J.P.M. using a chicken leptin radioimmunoassay (see below). 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. The primary antibody, rabbit anti-*rcleptin*, was purchased from a commercial source (Alpha Diagnostic International, San Antonio, TX). Iodination of *rcleptin* was conducted as previously described (McMurtry et al., 1994) with minor modifications. Aliquots of 10 μ g of *rcleptin* were dissolved in 25 μ L sodium phosphate buffer (0.5 M, 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 μ g) was added in 10 μ L sodium phosphate buffer (0.05 M, pH 7.4), and the reaction was stopped after 1 min by the addition of 20 μ g sodium metabisulphite (Fluka Chemical Corp., Ronkonhoma, NY) in 20 μ L sodium phosphate buffer (0.05 M). Un-reacted I-125-iodine was separated from I-125-labeled *rcleptin* by chromatography on Sephadex G-50 (Pharmacia, Piscataway, NJ) in 0.7×50 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, labeled *rcleptin* was aliquoted and stored at -80 °C.

Sodium phosphate buffer (0.05 M phosphosaline, pH 7.4), containing 0.025 M 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 rleptin) 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 μ L) plus a similar volume of standard or plasma unknown were added to plastic tubes containing 100 μ L of first antibody (rabbit anti-chicken leptin; 1:1600 working dilution), vortexed and incubated overnight at 4 °C. On day 2, 100 μ L tracer containing 6000 c.p.m. of I-125-labeled rleptin was added to each tube, vortexed and incubated overnight at 4 °C. On day 3, second antibody (100 μ L of a 1:10 dilution of sheep anti-rabbit gamma globulin) and carrier (100 μ L 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.

The displacement curve for starling plasma was parallel to the standard curve derived using chicken leptin (Fig. 1). We compared the slopes of these curves using proc MIXED (SAS Institute Inc., 2002) and analyses of covariance was unable to detect statistical differences between the standard slope (-0.0223) and the starling plasma slope (-0.0296 ; $P > 0.05$). Sensitivity of the leptin assay was determined to be 300 pg/tube. We determined recovery for chicken and starling plasma by spiking plasma pools with known amounts of recombinant chicken leptin (Table 1). Mean recovery of added leptin averaged 97.9% from chicken plasma and 96.1% from starling plasma. Unpublished (J.P.M.) work has shown that the chicken leptin antibody does not cross-react with chicken insulin, glucagon or GLP-1. Intra- and interassay coefficients of variation were calculated as described by Abplanalp et al. (1977) and were respectively 3.2% (mean concentration = 17.4 ng/mL; $n = 104$) and 5.1% (mean concentration = 8.7 ng/mL; $n = 6$).

2.3. Statistical analysis

All data were analyzed using 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 ($n_{\text{leptin}} = 57$ females total). Normality was evaluated using univari-

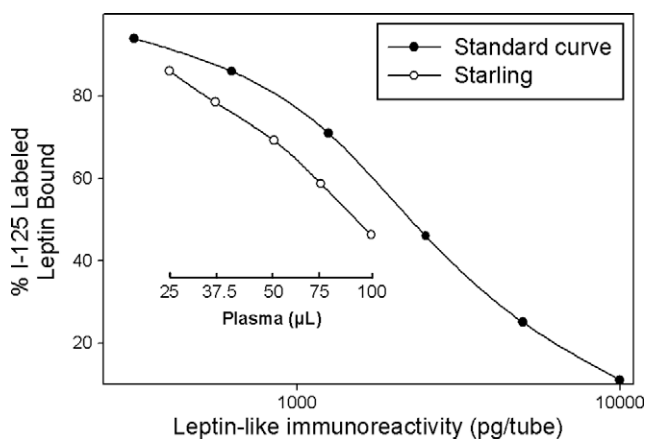


Fig. 1. Displacement curve for determination of specificity of chicken leptin RIA for starling plasma. Competitive binding curves were obtained with recombinant chicken leptin (●) and European starling (*Sturnus vulgaris*) plasma (○), and cross-reactivity was determined by adding increasing concentrations of starling plasma (25, 37.5, 50, 75, and 100 μ L) to the assay tubes. See text for statistical comparison of slopes.

Table 1

Recovery of recombinant chicken leptin in spiked chicken and European starling (*Sturnus vulgaris*) plasma. Recombinant chicken leptin was dissolved in 5 μ L saline and was added in varying amounts (ng/tube) to 95 μ L chicken ($n = 5$) or starling plasma ($n = 5$) pools. Values (ng/mL) are means \pm SEM.

Recombinant chicken leptin (ng/tube)	Chicken plasma leptin-like immunoreactivity (ng/mL)	Starling plasma leptin-like immunoreactivity (ng/mL)
0	8.4 \pm 1.1	19.3 \pm 1.6
2.5	10.6 \pm 1.3	20.8 \pm 1.9
5	13.5 \pm 0.9	23.9 \pm 2.4
10	19.1 \pm 1.7	30.8 \pm 2.7

ate analyses of raw data for correlations and regressions and using residuals for ANOVAs (Shapiro–Wilks). All of the analyzed traits approximated normality; therefore, parametric tests were used for all analyses. We analyzed the effect of breeding and non-breeding stages on variation in plasma leptin-like immunoreactivity, body mass, fat mass (exclusively in the 1999–2001 breeding seasons), and body condition (mass/tarsus) using general linear models (proc GLM). Post-hoc tests for differences between means were corrected for multiple comparisons using Tukey–Kramer adjustment. Pearson correlations and a regression were used to analyze

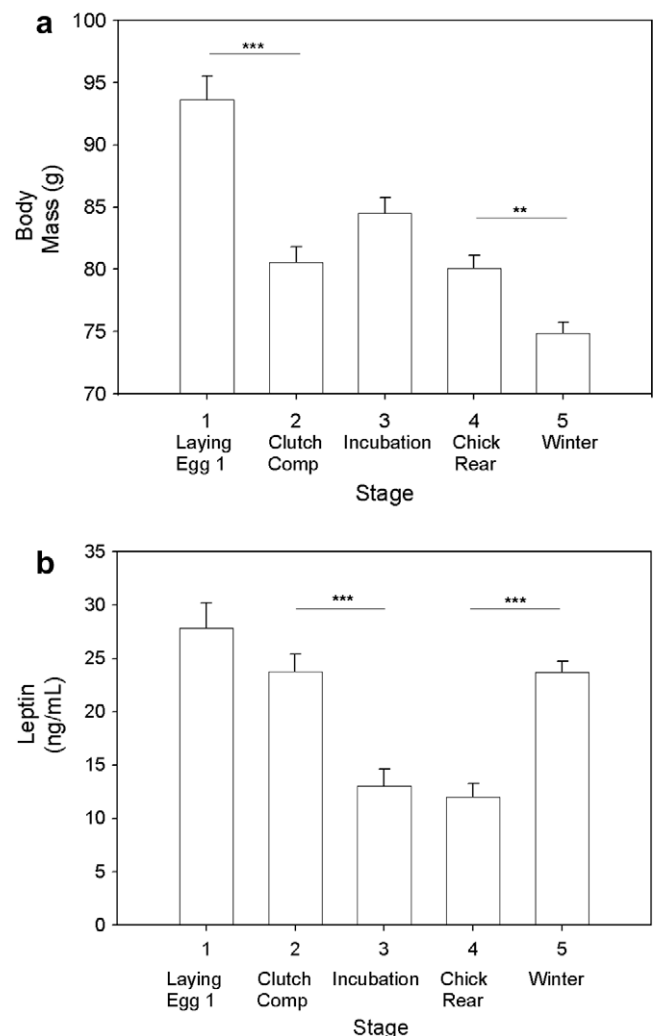


Fig. 2. Variation in (a) body mass (g); and (b) plasma leptin-like immunoreactivity (ng/mL) by stage (breeding and fall) among free-living female European starlings (*Sturnus vulgaris*). Asterisks between bars indicate significant differences at $P < 0.01$ level (**) and $P < 0.001$ level (***).

continuous variation in plasma leptin-like immunoreactivity in relation to residual (stage-specific) body mass, body condition, and to Julian date for pooled stages. All values are presented as least square means \pm SE unless otherwise stated.

3. Results

3.1. Seasonal variation in body mass and condition

Body mass varied by stage in 2007 ($F_{4, 51} = 24.35$, $P < 0.0001$; Fig. 2a); mass was highest during egg-laying and decreased by clutch completion ($P < 0.0001$), but then did not vary significantly through to chick-rearing. Body mass in non-breeding females in November 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 non-breeding females 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 marginally significantly lower than during chick-rearing ($P < 0.055$).

To confirm that the variation in mass and condition data during the 2007 breeding season was “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, unpublished data, Fig. 3). Analysis of variation in body mass by stage using the two data sets (2007 and 1999–2001) showed a dataset \times stage interaction ($F_{3, 215} = 5.08$, $P = 0.002$). This interaction occurred because although egg-laying and clutch completion body masses did not differ 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 for the 1999–2001 pooled data. 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. 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. 3 and 4a), and decreased after

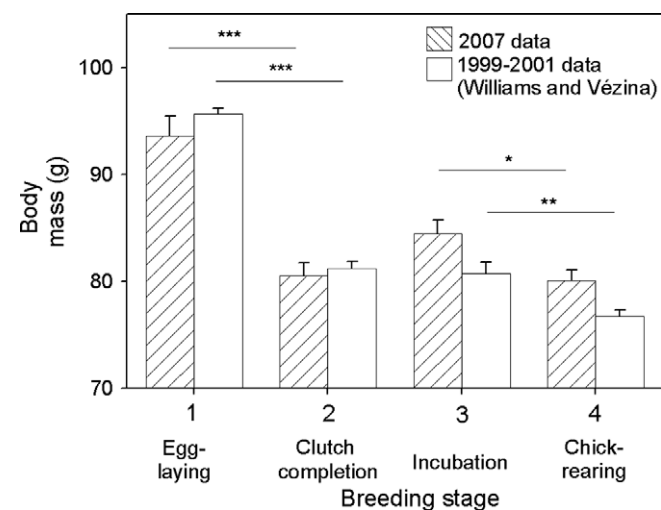


Fig. 3. A comparison of free-living female European starling (*Sturnus vulgaris*) body mass data (g) by breeding stage in the 2007 breeding season ($F_{3, 32} = 15.38$, $P < 0.0001$) and the pooled 1999–2001 breeding seasons ($F_{3, 156} = 218.91$, $P < 0.0001$). Asterisks between bars indicate significant differences at $P < 0.05$ level (*), $P < 0.01$ level (**), and $P < 0.001$ level (***).

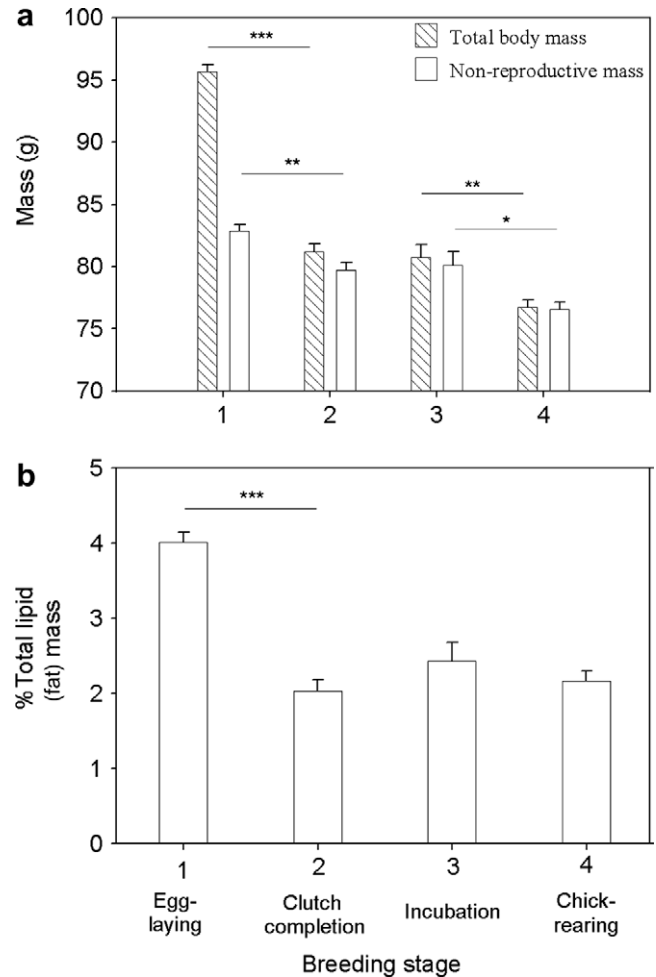


Fig. 4. Pooled data from free-living female European starlings (*Sturnus vulgaris*) during the 1999–2001 breeding seasons (Williams and Vézina, unpublished): (a) total body mass and non-reproductive mass (g) by stage; and (b) % of total fat mass by stage. Asterisks between bars indicate significant differences at $P < 0.05$ level (*), $P < 0.01$ level (**), and $P < 0.001$ level (***).

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 clarify 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. 4a) and was highest during egg-laying and decreased at 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. 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 then remaining constant through to chick-rearing ($P > 0.79$).

3.2. Seasonal variation in plasma leptin-like immunoreactivity

Plasma leptin-like immunoreactivity varied by stage ($F_{4, 52} = 21.11$, $P < 0.0001$; Fig. 2b). Plasma leptin-like immunoreactivity was not different in egg-laying females from those at clutch completion, but decreased significantly from clutch completion to incubation ($P = 0.0002$), remaining low during chick-rearing. We had data on a small number of females sampled in two breeding stages, and changes in plasma leptin-like immunoreactivity in these individuals confirmed the population-level pattern (Fig. 5). Plasma leptin-like immunoreactivity in non-breeding birds in

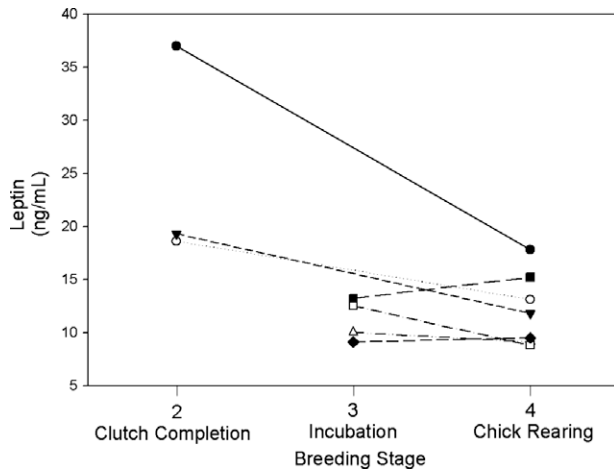


Fig. 5. Plasma leptin-like immunoreactivity values (ng/mL) for 7 repeat sampled free-living female European starlings (*Sturnus vulgaris*) during the 2007 breeding season by stage.

November 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 (using residuals controlling for stage), residual plasma leptin-like immunoreactivity was independent of both residual body mass ($r = -0.065$, $n = 56$, $P > 0.63$; Fig. 6a), and residual body condition ($r = 0.040$, $n = 56$, $P > 0.76$; Fig. 6b). Furthermore, the stage-specific variation in plasma leptin-like immunoreactivity remained significant when controlling for body mass ($F_{4, 50} = 21.00$, $P < 0.0001$); mass-corrected plasma leptin-like immunoreactivity decreased significantly after clutch completion ($P = 0.0006$), and mass-corrected plasma leptin-like immunoreactivity in non-breeding females was 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), $P < 0.0001$) due to the confound of stage-specific changes in leptin-like activity. However, within each of the breeding stages, sampling date did not affect plasma leptin-like immunoreactivity ($P > 0.11$ in all cases).

4. Discussion

In this study we describe, for the first time, variation in plasma leptin-like immunoreactivity in relation to reproductive stage and season in a free-living avian species. Plasma leptin-like immunoreactivity was highest during egg-laying and clutch completion in female European starlings, significantly decreased during incubation, and remained low during chick-rearing in breeding birds, a pattern also seen in repeat sampled individuals. In addition, we also found elevated levels of plasma leptin-like immunoreactivity in non-breeding females in November that were higher than in incubating and chick-rearing females but not different from levels at egg-laying and clutch completion. This suggests a seasonal, as well as breeding stage, component to variation in plasma 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. For example, the main change (a decrease) in body mass and fat mass occurred between egg-laying and clutch completion,

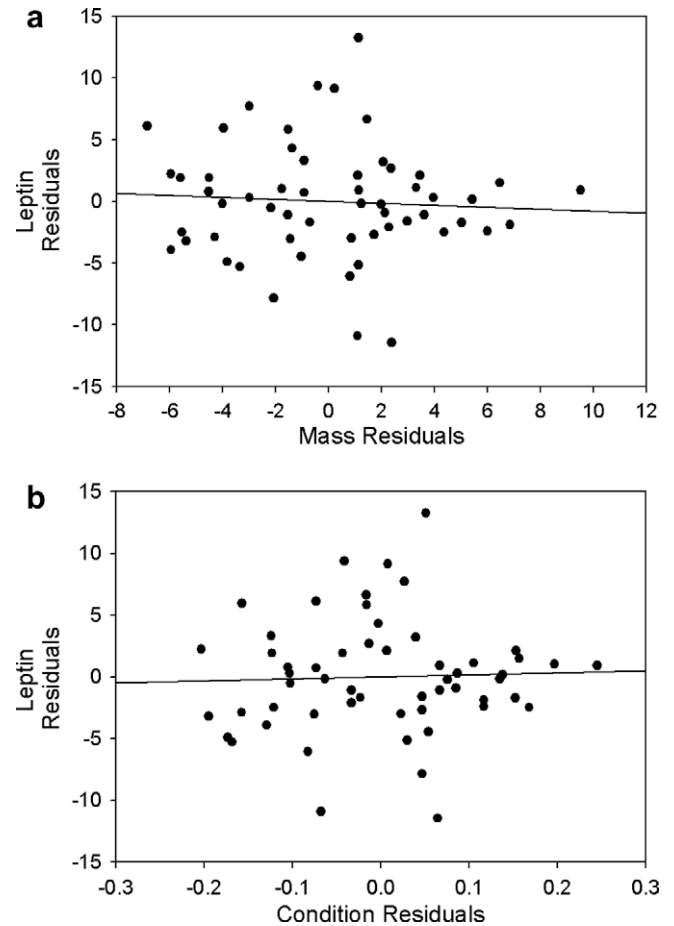


Fig. 6. Correlations of (a) residuals of plasma leptin-like immunoreactivity with residuals of mass when controlling for stage ($r = -0.065$, $P > 0.63$); and (b) residuals of plasma leptin-like immunoreactivity with residuals of condition (mass/tarsus) when controlling for stage ($r = 0.040$, $P > 0.76$) among free-living female European starlings (*Sturnus vulgaris*) during the 2007 breeding season. Individuals pooled across breeding stages and fall ($n = 56$).

while plasma leptin-like immunoreactivity remained constant at this time. Similarly, elevated plasma leptin-like immunoreactivity levels in non-breeding birds in November coincided with the lowest body masses recorded. Furthermore, overall, neither body mass nor body condition was correlated with plasma leptin-like immunoreactivity. These data suggest 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.

Our results contrast with numerous previous studies in mammals (Considine et al., 1996; Halaas et al., 1995) and in captive or domesticated quail and chickens (Lohmus et al., 2006; Shi et al., 2006) that have shown strong relationships between plasma leptin or leptin-like immunoreactivity and body mass or percent body fat. One potential explanation for these seemingly contradictory results relates to differences in the range of body masses in the respective studies. While there is an extensive range of body masses and adiposity in many mammalian species as well as in poultry species, the range of body fat among free-living starlings observed in our population is extremely narrow by comparison. For example, in humans plasma leptin is correlated with percent body fat over a range of approximately 5–65% (Considine et al., 1996). Similarly, the difference in abdominal fat mass between recombinant chicken leptin-immunized hens (115.6 g) and control hens (78.0 g) was 37.6 g; thus, treated hens had 50% more abdominal fat mass than control hens (Shi et al., 2006). In contrast, among

our free-living European starlings, total percent lipid mass only varied on average between 2% and 4% of body mass during the reproductive season and total body mass only varied from 70 to 100 g. This suggests that the marked, predominant relationship between leptin-like immunoreactivity and body or fat mass reported in many previous studies might not be typical of free-living birds. Similar to our findings, Quillfeldt et al. (2009) found no correlation between plasma leptin-like protein levels and body condition among free-living nestling thin-billed prions (*Pachyptila belcheri*). This highlights the value of investigating leptin-like function in free-living species with very different life-history characteristics from captive, domesticated species.

In breeding female European starlings, plasma leptin-like immunoreactivity was highest during the egg-laying phase of reproduction. Numerous studies have suggested that leptin-like activity is functionally related to different aspects of ovarian function and egg production in birds and we suggest that this might explain the high levels of leptin-like immunoreactivity in egg-laying females in our study. For example, leptin receptors have been identified in the hen ovary (Paczoska-Eliasiewicz et al., 2003) and variation in leptin concentration affects cell proliferation, apoptosis and secretory steroid hormone activity in cultured chicken ovarian cells (Sirotkin and Grossmann, 2007). The elevated levels of plasma leptin-like immunoreactivity in egg-laying females that we report in this study coincide with elevated plasma estradiol levels which occur during egg production in female birds (Dawson, 1983; Sockman and Schwabl, 1999). In addition, immunization against leptin has been shown to decrease laying rate among hens (Shi et al., 2006) and exogenous leptin treatment advances puberty in the domestic hen (Paczoska-Eliasiewicz et al., 2006). Consistent with this functional explanation, the liver is the primary site of vitellogenesis (lipogenesis) during egg production in birds, and this might provide a rationale for the observed hepatic expression of leptin-like activity in birds, unlike in mammals (Ashwell et al., 1999; Dridi et al., 2005; Kochan et al., 2006; Taouis et al., 1998).

The elevated levels of plasma leptin-like immunoreactivity we observed in non-breeding females must clearly have a different (non-reproductive) functional explanation, though this remains unclear at present. Several studies have demonstrated seasonal variation in serum leptin among some captive mammals including blue foxes, woodchucks and European brown bears (*Ursus arctos arctos*), and these seasonal changes in serum leptin generally follow patterns of body fat gain and loss (cf. our result). However, some studies have documented seasonal changes in leptin that do not directly correspond to fluctuations in body mass or adiposity (Kronfeld-Schor et al., 2000; Nieminen et al., 2001, 2002). For example, in the raccoon dog (*Nyctereutes procyonoides*), body mass increases between August and October and then remains high in preparation for winter sleep, and plasma leptin levels follow this pattern, but plasma leptin levels drop dramatically for one week in early November before rising again, even though body mass does not decrease at this time (Nieminen et al., 2002). Similarly, Mustonen et al. (2005) found that although plasma leptin levels in the blue fox peaked in mid October, individuals continued to accumulate body mass for several weeks after this. Both studies suggested that dissociation of plasma leptin and body mass might involve a period of leptin insensitivity (Mustonen et al., 2005; Nieminen et al., 2002). In pre-hibernatory little brown bats (*Myotis lucifugus*) changes in adiposity do not correspond to changes in plasma leptin levels (Kronfeld-Schor et al., 2000), and in *Sceloporus undulatus* lizards body size does not explain variation in plasma leptin levels (Spanovich et al., 2006). Non-breeding birds in winter may have higher energetic demands due to increased foraging or thermoregulation. However, since serum leptin concentrations should positively correlate with amounts of adipose tissue (e.g. Considine et al., 1996), one would have predicted low leptin-like

immunoreactivity levels among the November sampled starlings in this study. Similarly, limited evidence suggests there is a negative relationship between leptin and thermogenesis, e.g. 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), and in wild Mongolian gerbils (*Meriones unguiculatus*), serum leptin levels are lower in the winter than during the other seasons (Zhang and Wang, 2007b). Unfortunately, there is currently no research available on the effects of cold temperatures on leptin-like activity in birds. Although we are currently unable to explain this aspect of seasonal variation in plasma leptin-like immunoreactivity, we believe, again, that these data suggesting a strong seasonal pattern highlight the value of extending studies of leptin-like function to free-living, non-mammalian taxa.

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