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Short Communication

Circulating leptin levels do not reflect the amount of body fat in the dunlin *Calidris alpina* during migration

Patrycja Gogga [a,*], Joanna Karbowska [b], Zdzislaw Kochan [b,1], Wlodzimierz Meissner [a,1]

[a] Avian Ecophysiology Unit, Department of Vertebrate Ecology and Zoology, University of Gdansk, Wita Stwosza 59, 80-308 Gdansk, Poland
[b] Department of Biochemistry, Medical University of Gdansk, Debinki 1, 80-211 Gdansk, Poland

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ABSTRACT

Leptin is a peptide hormone that plays an important role in the regulation of energy homeostasis. Studies in mammals have shown that circulating leptin levels reflect adiposity and that this adipocyte-derived cytokine acts as an afferent satiety signal to the brain, decreasing food intake and increasing energy expenditure. Since leptin has been found in the liver and adipose tissue of migratory birds that are able to accumulate fat reserves as endogenous fuel for flight, we hypothesized that individuals with higher fat score would have higher plasma leptin levels, as it had been found previously in mammals. The aim of this study was to determine if circulating leptin levels correlate with the amount of body fat in a migratory bird, the dunlin *Calidris alpina*. Adult dunlins were caught during autumn migration on the Baltic coast, and their fat score was determined. Blood samples from 150 birds were used to assess the levels of circulating leptin. We did not find any statistical differences between dunlins with various fat scores. In fact, plasma leptin levels tended to be lower in fat birds than in lean individuals. Our data indicate that in wild birds in migration mode leptin does not reflect the amount of accumulated fat. It suggests that leptin in birds during migration is neither involved in the regulation of energy homeostasis nor acts as a signal to control the amount of body fat.

1. Introduction

Leptin is a small (16 kDa) peptide hormone, which in mammals is synthesized predominantly in white adipose tissue (Zhang et al., 1994) and, to a lesser extent, in bone marrow (Laharrague et al., 1998), muscles (Wang et al., 1998), brain (Morash et al., 1999), stomach (Bado et al., 1998), placenta (Senaris et al., 1997) and mammary gland (Smith-Kirwin et al., 1998). Leptin has also been found in other vertebrates such as fish (Kurokawa et al., 2005), amphibians (Boswell et al., 2006; Crespi and Denver, 2006), reptiles (Paolucci et al., 2011) and birds (Ashwell et al., 1999a; Kochan et al., 2006a). Leptin signaling is mediated by a specific membrane receptor, LEPRb, that is expressed in the hypothalamus and various peripheral tissues, such as intestine, liver, pancreas, ovary, testis, kidney, lung, heart and skeletal muscles (Fruhbeck et al., 1999). Leptin may act on peripheral tissues not only directly but also via central nervous system (Buettner et al., 2008; Gogga et al., 2011). The physiological role of leptin has been widely studied in rodents and humans (Reidy and Weber, 2000). In mammals, circulating leptin levels are highly dependent on nutritional conditions and are proportional to the amount of body fat (Kochan et al., 2006b; Zhang et al., 2002). This adipocyte-derived protein acts as a satiety hormone that decreases food intake and increases energy expenditure, thus regulating energy homeostasis (Belgardt and Bruning, 2010). However, in some organisms, such as seasonal mammals and pregnant rats, leptin resistance has been observed, as a physiological adaptation to specific environment conditions (Tups, 2009). In addition to its role in metabolic control, leptin modulates the activity of a number of physiological systems, such as immune (Lord et al., 1998; Lord, 2002), vascular (Boulomie et al., 1998) and reproductive (Clarke and Henry, 1999).

Despite reports of cloning and sequencing leptin gene in birds (Taouis et al., 1998; Ashwell et al., 1999a), some researchers failed to confirm its existence (Friedman-Einat et al., 1999; Ninov et al., 2008; Yosefi et al., 2010) and deny leptin expression in these vertebrates. Additionally, the controversy concerns very high similarity (97%) in nucleotide sequence between chicken and mouse leptin (Taouis et al., 1998), which is very unlikely from an evolutionary point of view. To date, no leptin gene has been found in avian genome. It has even been proposed that in birds the gene encoding leptin has been lost during the course of evolution (Pitel and Faraut, 2010). On the other hand, there are several reports demonstrating the presence of leptin (Taouis et al., 1998; Ashwell et al., 1999a; Kochan et al., 2006a; Quillfeldt et al., 2009) and its receptor (Richards and Poch, 2003; Liu et al., 2007; Ohkubo et al., 2007; Cerasale et al., 2011) in wild and domestic birds. Assays

* Corresponding author. Fax: +48 58 523 61 44.
E-mail address: aviridis@gmail.com (P. Gogga).
[1] These authors share senior authorship.

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based on antibody-antigen interactions enable detection of leptin or at least leptin-like protein, which is very similar in size and structure (Dridi et al., 2000a; Kochan et al., 2006a; Neglia et al., 2007; Quillfeldt et al., 2009; Kordonowy et al., 2010). Leptin-induced activation of the JAK-STAT pathway (the main signaling pathway initiated by leptin in mammals) has also been demonstrated (Adachi et al., 2008). Moreover, it appears that in birds, as in mammals, leptin is involved in the regulation of energy metabolism. For instance, leptin administration reduces food intake in the chicken (Denbow et al., 2000; Dridi et al., 2000b, 2005). Anorexigenic effect of leptin has also been observed in wild species, the great tit *Parus major* (Lohmus et al., 2003) and the Asian blue quail *Coturnix chinensis* (Lohmus et al., 2006). It suggests that in birds this hormone signals nutritional status to the central nervous system. If energy metabolism in migrating bird species was controlled by the same mechanism as in mammals, the individuals with higher fat load should have higher levels of plasma leptin. This would lead to a decrease in food intake and inhibit fattening. In fact, birds before a migratory flight are able to accumulate fat deposit up to nearly 50% of their body mass (Gill et al., 2005), so one can assume that they somehow circumvent satiety signal related to leptin. This study was aimed to investigate if levels of circulating leptin in a migratory bird, the dunlin *Calidris alpina,* reflect the amount of accumulated fat reserves, as in mammals. Given the controversy surrounding the avian leptin, in this study we use the term "leptin", but we mean "leptin-like protein".

2. Materials and methods

2.1. Fieldwork

Dunlins were caught in walk-in traps (Meissner, 1998a) on the southern Baltic coast, in the Vistula river mouth (54°21.44′N, 18°56.61′E). Shorebirds stop in this area to accumulate energy reserves during migration toward wintering grounds (Gromadzka, 1998). Dunlins were aged according to plumage features (Meissner and Skakuj, 2009). The body mass (accuracy 1 g) and bill length (accuracy 0.1 mm) of caught birds were measured to assess mass of accumulated fat according to predictive equation given by Meissner (1998b). Blood samples (10–50 μl) were collected from 150 adult dunlins (older than 1 year) from the brachial vein and centrifuged to obtain plasma, which was immediately frozen and stored in liquid nitrogen. Samples were transferred to the laboratory and stored at −70 °C until analyses. Samples were collected between the last week of July and mid-August in 2009, and during the last week of July and the first week of August in 2010.

The amount of fat reserves in caught birds has been scored by visual assessment of subcutaneous fat deposit according to fat score scale developed for shorebirds (Meissner, 2009). For further analyses birds were divided into three groups (Table 1):

- lean individuals – fat scores 0 or 1,
- medium fat individuals – fat scores 2 or 3,
- fat birds – fat score 4 or higher.

Among dunlins caught at this stage of migration adiposity categories are not evenly distributed and majority of birds usually depart from this area with low or medium energy reserves (Meissner, 1998b). Thus, the group consisting of the fattest individuals was smaller than the other groups and represented mainly by birds with fat scores 4 or 5, while fat scores 6 or 7 were observed only in single individuals.

Table 1
Number of sampled birds and plasma samples used in the study.

Fatness group	Range of fat scores	Mean body mass [g] ± SD	Number of sampled birds	Number of analyzed plasma samples
Lean	0–1	41.4 ± 3.60	56	18
Medium	2–3	44.9 ± 3.32	51	16
Fat	4–7	51.3 ± 4.40	43	14

2.2. Radioimmunoassay (RIA) of plasma leptin

Plasma leptin levels were determined using the multispecies leptin RIA kit (Millipore, Billerica, MA, USA). Assays were performed according to the manufacturer's instruction, with slight modifications to adjust the procedure to small sample volumes. Because little amount of blood was collected from some individuals, plasma samples from birds of the same fatness group were pooled, so one sample consisted of plasma from one to five birds. We made an additional standard sample (0.5 ng/ml of leptin) to increase precision of the standard curve.

2.3. Western blot analysis

Protein levels of leptin were determined in dunlins' plasma using specific polyclonal antibodies. Aliquots of plasma (4 μl) diluted in loading buffer containing 2% sodium dodecyl sulfate (SDS) and 50 mM dithiothreitol were boiled for 5 min and separated by SDS–polyacrylamide gel electrophoresis in Any kD gradient gels (Bio-Rad, Hercules, CA, USA), followed by transfer to Hybond membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). To check transfer of proteins and assess the uniformity of loading, the membrane was stained with Ponceau S (Sigma, St. Louis, MO, USA). After destaining, the membrane was blocked with 5% skimmed milk in PBS-T buffer (Phosphate Buffered Saline, pH 7.4, 0.1% Tween 20) for 2 h at room temperature. Next it was incubated with rabbit anti-leptin polyclonal antibodies (Pierce, Rockford, IL, USA), followed by HRP-conjugated secondary antibodies (Sigma) (Kochan et al., 2006a). Primary antibodies were raised against a synthetic peptide corresponding to N-terminal domain of mouse leptin, which is highly conserved among vertebrates (Boswell et al., 2006). The concentration of leptin in the blood is in the low nanogram per milliliter range, for example serum leptin levels in fasted rats are below 2 ng/ml (Karbowska and Kochan, 2012). Having in mind that leptin may be present in low concentrations in the blood of dunlins, proteins on the membrane were visualized using SuperSignal West Pico substrate (Pierce), which is an enhanced chemiluminescent substrate and provides low picogram detection of proteins in Western blot applications. The membranes were exposed to Kodak XAR film (Kodak, Rochester, NY, USA) for 2–10 min.

2.4. Statistical analyses

Mean levels of plasma leptin in birds from different fatness groups were compared by single factor ANOVA using STATISTICA 9.1 software (StatSoft, 2010). The systematic trend in plasma leptin levels across fatness groups was checked using Jonckheere–Terpstra trend test (Jonckheere, 1954). To examine the possible correlation between plasma leptin levels and fat mass Spearman's correlation coefficient was used.

3. Results

Mean fat mass of analyzed birds from three fatness groups differed significantly (ANOVA, $F_{2,39} = 104.32$, $P < 0.001$) and high differences were observed between all groups (Tukey post hoc test, $P < 0.05$) (Fig. 1).