Lack of leptin activity in blood samples of Adélie penguin and bar-tailed godwit

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Abstract

Unsuccessful attempts to identify the *leptin* gene in birds are well documented, despite the characterization of its receptor (LEPR). Since leptin and LEPR have poor sequence conservation among vertebrates, we speculated that a functional assay should represent the best way to detect leptin in birds. Using a leptin bioassay that is based on activation of the chicken LEPR in cultured cells, blood samples from wild birds with extreme seasonal variation in voluntary food intake and fat deposition (Adélie penguins and bar-tailed godwits) were tested for leptin activity. In these experiments, blood samples collected during the

pre-incubation and the chick-rearing periods of Adélie penguins, and during the migratory flight and refueling stages of bar-tailed godwits, were found to contain no detectable leptin activity, while the sensitivity of the assay to activation by human blood samples from donor subjects representing a variety of body mass indices and fat contents was clearly demonstrated. These results suggest that in birds, an alternative control mechanism to that of mammals operates in the communication between the body fat tissues and the central control on energy homeostasis.

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Introduction

In mammals, the satiety hormone leptin (Zhang *et al.* 1994) is secreted primarily by the fat tissue and has proven to be a key regulator of energy metabolism (Friedman 2002, Farooqi & O'Rahilly 2009). Leptin signals the amount of fat stores to the hypothalamus and peripheral tissues, which then modulate their activity according to energy status. The more recent identification of leptin and leptin receptor (LEPR) in non-mammalian vertebrates, such as fish (Johnson *et al.* 2000, Huising *et al.* 2006, Kurokawa *et al.* 2008, Yacobovitz *et al.* 2008, Kurokawa & Murashita 2009) and amphibians (Boswell *et al.* 2006, Crespi & Denver 2006), and the identification of LEPR in chickens (CLEPR) and in turkeys by us and others (Horev *et al.* 2000, Ohkubo *et al.* 2000, Richards & Poch 2003, Liu *et al.* 2007) strongly suggest the presence of leptin in birds. Expression of the CLEPR in brain and other tissues was demonstrated at both the mRNA (Horev *et al.* 2000, Ohkubo *et al.* 2000, Richards & Poch 2003, Dridi *et al.* 2005b, Liu *et al.* 2007, Adachi *et al.* 2008, Byerly *et al.* 2009, Yuan *et al.* 2009) and the protein (Ohkubo *et al.* 2007) levels. We previously mapped the CLEPR to a chromosomal site

syntenic to that in humans (Dunn *et al.* 2000). In addition, we showed that when introduced into HEK-293 cells together with a signal transducers and activators of transcription 3 (STAT3)-derived reporter gene, CLEPR specifically activates the reporter gene in response to leptin (Hen *et al.* 2008). Specific STAT3 phosphorylation and activation of the Janus kinase (JAK)–STAT pathway by CLEPR *in vitro*, similar to the signal transduction pathway characterized in mammals, were demonstrated in several cell culture systems (Adachi *et al.* 2008). In addition, the recombinant predicted leptin-binding domain of CLEPR was shown to specifically bind leptins of several origins *in vitro* (Niv-Spector *et al.* 2005).

Altogether, these demonstrations of conservation of CLEPR with respect to its structure, chromosomal position, expression pattern and biological activity (Dunn *et al.* 2000, Horev *et al.* 2000, Ohkubo *et al.* 2000, Richards & Poch 2003, Niv-Spector *et al.* 2005, Liu *et al.* 2007, Adachi *et al.* 2008, Hen *et al.* 2008) could be considered as an indication of its function *in vivo*. However, the CLEPR ligand has not yet been found (Friedman-Einat *et al.* 1999, Dunn *et al.* 2001, Amills *et al.* 2003, Sharp *et al.* 2008, Pitel *et al.* 2009). In addition, despite the nearly complete sequencing of the chicken genome

(Burt 2006) and extensive sequencing of chicken fat expressed sequence tags (ESTs) (Cogburn *et al.* 2003), no sequence similarity to leptin has been identified, nor has any similarity been found at the protein level (Sharp *et al.* 2008).

Our hypothesis was that since leptin is among the class-I helical cytokines with the poorest sequence conservation in vertebrates (Huising *et al.* 2006), a functional assay might be a more appropriate way of detecting it, compared with assays depending on sequence similarity. In a previous report (Hen *et al.* 2008), we described a chicken leptin bioassay in cultured HEK-293 cells based on the exogenous expression of CLEPR and a luciferase reporter gene. The sensitivity of this assay system to various heterologous leptins of *Xenopus* and mammalian origin was demonstrated, but we failed to detect specific leptin activity in blood samples from commercial chicken strains with lean or fat phenotypes (Hen *et al.* 2008). One of the hypotheses to explain the absence of leptin in these commercial chicken strains was that it had disappeared in the course of intensive genetic breeding toward high production yields.

Therefore, the presently described study focused on detecting leptin activity in blood samples of wild birds. Adélie penguin (*Pygoscelis adeliae*) and the bar-tailed godwit (*Limosa lapponica*) are very different birds, but both transiently accumulate exceptionally high amounts of fat, to over 20–30% of their body weight (BW), which is followed by extreme voluntary periods of food withdrawal (incubation and migration respectively). The hypothesis was that if leptin is the satiety hormone in birds, it should have a dominant role in the extreme seasonal differences in appetite and fat accumulation in these birds.

The breeding cycle of the Adélie penguin comprises four phases: i) the courtship from mid-October to early November; ii) the incubation of one or two eggs for 30–36 days; iii) the guard stage (from mid-December to mid-January) when both parents alternate between foraging at sea and chick attendance at the nest, and iv) the crèche stage (from mid-January to mid-February), when both parents can forage at the same time leaving the chick(s) alone in the colony. By using body mass and total body water content being measured by isotope dilution, Chappell *et al.* (1993) determined the body composition of Adélie penguins and precisely described how body mass, depot fat, and lean tissue simultaneously change along the breeding cycle. When they arrive on the colony to breed at the beginning of the austral summer, Adélie penguins are heavy (males: 4.5–5.5 kg and females: 4.0–4.5 kg), and depot fat represents 20–30% of the penguins' body mass. During the incubation fast, penguins' body mass decreases mainly through fat loss (and to a lesser extent through lean tissue loss). Consequently, depot fat decreases sharply during the incubation stage but remains low and stable (7–10%) from the end of the incubation through the chick-rearing period in both males and females (Chappell *et al.* 1993).

The bar-tailed godwit is a migratory bird that performs the longest known non-stop migratory flight (11 500 km from Alaska to New Zealand (McCaffery & Gill 2001). Here, we

studied a subspecies of bar-tailed godwits (*L. lapponica taymyrensis*) during spring migration from wintering areas in West Africa to breeding grounds in northern Russia (ca. 9000 km). Godwits perform this migration in two non-stop flights of 2–3 days each (ca. 4500 km), stopping on the coast of the Wadden Sea in Germany, Denmark, and The Netherlands to refuel (Piersma & Jukema 1990). On arrival in the coast of the Wadden Sea, godwits are emaciated and are in a state of extreme fasting (see Landys-Ciannelli *et al.* (2002)). During their month-long stopover, godwits double their body mass and deposit large amounts of fat (i.e. over 30% of body mass), which constitute the main fuel for subsequent migratory flights (Landys *et al.* 2005) to Russia.

In this report, blood samples collected during the pre-incubation and guard stages of Adélie penguins, and the migratory flight and refueling stages of bar-tailed godwits were compared with human blood samples collected from donor subjects representing a variety of body mass indices (BMIs) and fat contents using a leptin bioassay that is based on activation of the CLEPR.

Materials and Methods

Cytokines

Human leptin was kindly donated by Prof. Arie Gertler (The Hebrew University, Rehovot, Israel). Interferon- α (IFN- α) was a kind gift from Dr Daniela Novic and Dr Meacham Rubinstein at the Weizmann Institute of Science, Rehovot, Israel.

Human blood sampling

Human blood samples were collected from volunteers by a registered nurse. The samples were divided into two aliquots for plasma and serum preparation. For serum preparation, the blood samples were left at room temperature for a few hours and then kept in the refrigerator overnight. For plasma preparation, the samples were transferred to heparinized tube in ice. After separation of serum/plasma at 1500 g centrifugation, each sample was divided into two aliquots: one was kept at -20°C and the other was dried by lyophilization. Lyophilized samples were restored to their original volumes with double-distilled water.

Adélie penguin seasonal cycle and blood sampling

Adélie penguins breed all around Antarctica from mid-October to mid-February. For this study, penguins were captured in the French station Dumont d'Urville ($66^{\circ}40' \text{ S}$, $140^{\circ}00' \text{ E}$) in the austral summer 2007–2008. Blood was collected from the wing vein with a heparinized syringe during the pre-incubation stage from nine females and seven males, and 40–45 days after egg-laying during the guard stage from eight females and eight males. Sex determination was

carried out by using a combination of parameters including cloacal inspection before egg-laying, copulatory behavior, and incubation routine (Taylor 1962, Kerry *et al.* 1993). After centrifugation, plasma and red blood cells were separated and stored at -20°C . After blood collection, each penguin was weighed with an electronic balance (Ohaus Corporation, Nänikon, Switzerland, ± 2 g). As expected, males were heavier than females (general linear model (GLM): $F_{1, 28} = 16.58$, $P < 0.001$), and penguins were about 9% heavier before the incubation than during the guard stage (4.81 ± 0.55 kg and 4.39 ± 0.44 (mean $\pm$ s.d.) respectively; GLM: $F_{1, 28} = 13.12$, $P < 0.001$); this percentage corresponds to the difference in body mass reported by Chappell *et al.* (1993) between these two breeding stages. Although the body mass difference between the pre-incubation and guard stages tended to be higher in males than in females, this was not significant (GLM: $F_{1, 28} = 3.40$, $P = 0.08$).

Bar-tailed godwit migratory route and blood sampling

Bar-tailed godwit blood samples were collected from birds at two sites. At the first site, 'arriving birds' were lured to land using song playback and decoys in the dunes near Castricum, The Netherlands ($52^{\circ}32' \text{N}$, $04^{\circ}37' \text{E}$), 60 km from their established stopover site in the Wadden Sea. At the second site, in Terschelling, The Netherlands ($53^{\circ}39' \text{N}$, $0^{\circ}32' \text{E}$), birds were captured during the middle of their refueling period as described before (Landys *et al.* 2005). After capture, body mass was measured on a digital balance (± 0.1 g), and wing length was measured with a wing ruler (± 0.5 mm). Whole blood was collected by brachial vein puncture. Plasma was separated by centrifugation at 1500 g, flash frozen in liquid nitrogen dry shippers, stored at -80°C , and lyophilized.

Leptin activity

Leptin activity was measured using a bioassay (Hen *et al.* 2008) consisting of HEK-293 cells expressing exogenous full-length CLEPR cDNA and a luciferase reporter gene under the control of a STAT3-responsive element, as described previously (Hen *et al.* 2008). Briefly, test and control cultures, harboring both CLEPR and reporter genes or the reporter gene only, respectively, were plated in 48-well tissue culture plates (Nunc, Danyel Biotech, Rehovot, Israel) at a concentration of 2×10^5 cells per well in a final volume of 200 μl . The following day, medium in each well was replaced with 150 μl DMEM (Gibco/BRL) or with medium containing purified proteins (leptin or IFN- α), or with medium containing 15 μl plasma samples for 4 h. Medium was then aspirated, and the cells were lysed by adding 100 μl Promega cell lysis reagent. A 40 μl aliquot of each cell lysate was mixed with 40 μl Promega luciferase assay agent. Luciferase activity was measured using the TD20e luminometer (Turner Design, Mountain View, CA, USA).

IFN- α activates the STAT3-responsive element similar to leptin. However, unlike leptin, IFN- α operates through an endogenous receptor. Therefore, IFN- α was used to demonstrate the presence of the inducible reporter construct in the control cell line. The response to IFN- α is expected to be similar in both the control and the CLEPR cell lines. However, on repeated freezing and thawing cycles, the control cells have acquired a higher sensitivity to IFN- α .

Estimation of fat contents

Fat contents in the human donors were estimated according to BMI values, taking into account age, gender (Jackson *et al.* 2002), and physical activity (Sempolska & Stupnicki 2007), and using the Linear Software at <http://www.linear-software.com/online.html>.

For Adélie penguins, fat content estimation was based on previous report (Chappell *et al.* 1993) using a detailed isotope dilution approach. In our study, body mass of the courtship (males: 5.27 ± 0.37 kg; females: 4.46 ± 0.38 kg) tightly corresponded to the body mass of penguins at arrival (Chappell *et al.* 1993; males: 5.10 ± 0.27 kg and females: 4.53 ± 0.35 kg), which were found to contain 28 and 22% body fat in males and females respectively. Similarly, fat content during the guard stage was estimated to be 10 and 8% of the body mass of males and females respectively (Chappell *et al.* 1993).

Body fat content of bar-tailed godwit was estimated based on formulas described previously (Piersma & Jukema 1990) correlating body fat mass, body mass, and wing length of bar-tailed godwit. The calculated values of fat mass for the birds presented here are within the range of fat mass from studies that have directly measured body fat content (Landys-Ciannelli *et al.* 2002).

Statistical analysis

Content data were analyzed by one-way ANOVA, Student's *t*-test, and Tukey–Kramer test. The analyses were performed using JMP 5.1 software (SAS Institute, Cary, NC, USA) at a significance level of $P \leq 0.05$.

Results

Analysis of leptin activity in blood samples from Adélie penguins

A bioassay, developed previously in our laboratory for the detection of leptin activity through activation of CLEPR in cultured cells (Hen *et al.* 2008), was used to monitor leptin activity in the plasma samples of Adélie penguins (Fig. 1). Although the human plasma sample, used as a positive control, significantly and specifically activated luciferase activity in the CLEPR-expressing cells, but not in the control cells, no such activity could be detected in the plasma samples from male and female penguins at the pre-incubation and guard stages (Fig. 1).

Figure 1 Absence of leptin activity in plasma samples from Adélie penguins. Plasma samples collected from Adélie penguins at the indicated seasonal periods were incubated with CLEPR-transfected cells and control cell lines, and induction of the reporter gene luciferase was measured as detailed in 'Materials and Methods' section. Recombinant human leptin (final concentration 30 ng/ml) and IFN- α (30 U/ml) were used as controls. IFN- α was used to demonstrate the presence of the inducible luciferase activity in the control cell line as detailed in 'Materials and Methods' section. Data are expressed as fold stimulation over culture medium alone and are means $\pm$ S.E.M. from seven to nine samples of the female (F) and male (M) at pre-incubation (pre-in) and guard stages, performed in duplicates; and from three independent assays for the leptin and IFN- α . Except for the recombinant human leptin and IFN- α , no other treatment exhibited significant induction at $P \leq 0.05$. * $P < 0.002$ compared with value for control cells. ** $P > 0.02$ compared with the values for control cells of the other treatments. *** $P > 0.05$ compared with the CLEPR cells treated with the bird's sample.

Analysis of leptin activity in samples from bar-tailed godwits

Blood samples from migratory flight and refueling stages of bar-tailed godwits were tested for leptin activity, and as can be observed in Fig. 2, no leptin-like activity was detected in the plasma samples collected at either seasonal stage. The same results were obtained when the bioassay was performed at 20% instead of 10% plasma concentration (not shown).

Activation of CLEPR by human blood samples

Leptin activity in the plasma samples from humans with variety of BMI values was tested in the CLEPR bioassay. As shown in Fig. 3, specific activation of CLEPR was observed with all of the samples, including those from donors with low BMI values. The relatively high-fold induction values obtained for the recombinant leptin and for the plasma samples from subjects with BMIs 32 and 23.5 are within the plateau phase of bioassay. To get the expected linear correlation between the leptin activity and the BMI values, each sample had to be repeated at several dilutions as described earlier (Friedman-Einat *et al.* 2003). However, more important is the finding that the plasma samples obtained from subjects with relatively low BMI values significantly activated CLEPR signal transduction.

In this assay, possible sensitivity to plasma lyophilization, which was used to preserve the birds' plasma samples, was found to have no effect on leptin activity. These control studies were repeated several times with several blood samples (representative results are shown in Fig. 3). The use of serum instead of plasma prepared from the same blood sample had no effect on leptin activity (data not shown).

To estimate the relevance of the human controls to the bird analyses, estimated proportions of body fat were used, based on the constituent correlation characterized in mammals between body fat and circulating leptin activity (Levine *et al.* 1999, Sagawa *et al.* 2002, Staiger *et al.* 2003, Kempf *et al.* 2006, Smith *et al.* 2006, Friedman 2009). As shown in Table 1, the estimated fat content of some of the penguins and bar-tailed godwits was within the range of the human samples that induced luciferase activity in the bioassay. The higher estimated fat stores in the BMI of female compared with the BMI 22 of male are compatible with the higher leptin activity in the corresponding blood samples as shown in Fig. 3.

Analysis of possible leptin inhibitory activity in the blood samples

To test for the possibility of leptin inhibitory activity in the birds' blood samples, leptin spike-in experiment was performed (Fig. 4). As demonstrated in Fig. 4, CLEPR-expressing cells responded to incubation with recombinant human leptin, regardless of the presence of penguin plasma. The recombinant human leptin similarly showed an additive effect when mixed with a human blood sample. This result suggests that there is no leptin-inhibiting activity in the penguin blood samples, which would inhibit the CLEPR activation in the bioassay.

Figure 2 Absence of leptin activity in plasma samples from bar-tailed godwit birds. Plasma samples were collected from bar-tailed godwit birds at the indicated seasonal periods. Samples were incubated with CLEPR-transfected cells and control cells as described in Fig. 1. Data are expressed as fold stimulation of the reporter gene luciferase over culture medium alone and are means $\pm$ S.D. from two independent experiments, performed in duplicate. Except for the signal obtained with recombinant human leptin (final concentration 30 ng/ml), no other treatment exhibited significant induction at $P \leq 0.05$. * $P < 0.002$ compared with value for control cells.

Figure 3 Leptin activity in human blood samples. Stimulation of luciferase activity in CLEPR and control cell lines by recombinant human (H) leptin (30 ng/ml) and by plasma samples from human subjects with different BMIs (L, lyophilized plasma sample). The assay was performed as described in Fig. 1. The values obtained for the recombinant leptin and for the BMIs 32 and 23.5 are within the plateau phase of bioassay. Data are expressed as fold stimulation of the reporter gene luciferase over culture medium alone and are means $\pm$ s.d. from two independent experiments, performed in duplicates. * $P < 0.05$ compared with value for control cells.

Discussion

Using a chicken leptin bioassay, which is based on the expression of CLEPR in cultured cells, we found for the first time the lack of leptin bioactivity in the blood samples of wild birds with extreme seasonal variation in voluntary food intake and fat deposition. These results were unexpected due to the characterization of the LEPR in chickens and turkeys (Horev *et al.* 2000, Ohkubo *et al.* 2000, Richards & Poch 2003), and the demonstration that these receptors have retained their leptin-binding and signal transduction activities (Niv-Spector *et al.* 2005, Adachi *et al.* 2008, Hen *et al.* 2008).

The use of rare blood samples obtained from Adélie penguins and bar-tailed godwits provided a crucial indication that, in birds, signaling the amount of fat stores is not mediated by leptin. This study strongly suggests that the previously reported lack of leptin activity in chickens (Hen *et al.* 2008) is not due to intensive genetic breeding of commercial chicken strains or to their relative leanness, but is also characteristic of wild birds in their natural habitat exhibiting voluntary seasonal changes in appetite and fat deposition.

Despite relatively low predicted amino acid sequence similarity (about 60%) between CLEPR and the mammalian orthologs (Horev *et al.* 2000, Ohkubo *et al.* 2000), a high degree of cross-activation of CLEPR by mammalian leptins has been previously reported by us and others (Adachi *et al.* 2008, Hen *et al.* 2008). The cross-species binding of CLEPR has been demonstrated both in modified cell cultures expressing exogenous CLEPR cDNA (Adachi *et al.* 2008, Hen *et al.* 2008) and by surface plasmon resonance assay using the recombinant leptin-binding domain of CLEPR (Niv-Spector *et al.* 2005). These results are compatible with the high similarity in predicted tertiary structure between the mammalian leptin and the highly divergent *Xenopus* leptin (Crespi & Denver 2006).

Physiological concentrations of circulating leptin in mammals range from about 3 to 80 ng/ml (Tomimatsu *et al.*

1997, Farooqi *et al.* 2001, Friedman-Einat *et al.* 2003, Nkrumah *et al.* 2007). We previously demonstrated that both the CLEPR cells described herein and human LEPR-expressing cells described in previous reports (Rosenblum *et al.* 1998, Marikovsky *et al.* 2002, Friedman-Einat *et al.* 2003, Hen *et al.* 2008) are sensitive to this range of leptin concentrations. The activation of CLEPR by human samples with various BMI values was expected, taking into account that a direct linear correlation between BMI values and circulating leptin levels is observed at the population level (Farooqi *et al.* 2001). Nevertheless, it is important to note that the CLEPR in our bioassay responded to incubation with blood samples from human donors with various BMI values including values below 25, which are indicative of normal BW and relatively low fattening. The comparison between the humans and birds used in this study was based on the estimations of their fat deposition. It is logical to assume that if circulating leptin levels in the birds signaled the amount of fat stores to the brain and some peripheral tissues in a manner similar to that in mammals, these leptin levels would be detected in the CLEPR-based bioassay.

The observed absence of leptin degradation or inhibiting activity in the birds' blood samples demonstrated here by spike-in experiments is compatible with a previous report showing that the rate of plasma clearance of infused mouse leptin is quite similar in broiler chickens (23 min; McMurtry *et al.* 2004) and the clearance of endogenous leptin in humans (25 min; Klein *et al.* 1996). The spike-in experiment described here also indicates that there is no circulating activity blocking leptin binding to CLEPR in the bioassay. A hypothetical leptin-blocking activity that is highly specific to a putative bird leptin cannot be excluded by our experiments. But this hypothesis seems unlikely because unless operating only *in vitro*, this chicken leptin-specific blocking activity would be expected to correspond to the bird's physiological state, allowing leptin signaling in a situation of high-fat content (or possibly other physiological states as discussed below).

Table 1 Estimated fat contents in the blood samples of donors

Stage/BMI and gender		Percentage of body fat
Human	BMI 32, F	34
	BMI 23.5, F	26
	BMI 22, M	18
	BMI 18, F	21
Penguin	Courtship, M	28
	Courtship, F	22
	Guard, M	10
Godwit	Guard, F	8
	Refueling T067	20
	Refueling T072	12
	Arriving C011	3
	Arriving C066	3

M, male; F, female.

Figure 4 Absence of leptin inhibitory activity in the bird's blood samples. Stimulation of luciferase activity in CLEPR cell line by recombinant human leptin (10 ng/ml) alone or in combination with plasma samples from female (F-guard) and male (M-guard) at guard stage and from female (F-pre-in) and male (M-pre-in) at pre-incubation stage. Data are expressed as fold stimulation of the reporter gene luciferase over culture medium alone and are means $\pm$ S.E.M. ($n=3$). * $P<0.01$ compared with the other leptin treatments. No significant difference was obtained between leptin in the plasma serum compared with leptin alone at $P<0.05$.

In mammals, changes in voluntary food intake and adiposity usually involve changes in leptin sensitivity. In obese humans, except in extremely rare cases, obesity is associated with decreased sensitivity to leptin and increased circulating leptin levels, a phenomenon that has been termed leptin resistance (Frederich *et al.* 1995, Caro *et al.* 1996, Ur *et al.* 1996, Montez *et al.* 2005). Leptin resistance can be either a pathological state, for example, in diet-induced obesity or an adaptive response allowing shifts in body-energy stores, similar to that occurring in seasonal breeders (Rousseau *et al.* 2002, Tups *et al.* 2004, Krol *et al.* 2007, Zieba *et al.* 2008, Scarpace & Zhang 2009, Tups 2009). For example, the Siberian hamster, which has been intensively studied as a model of seasonal mammals, spontaneously reduces its BW by $\sim 40\%$ over a 12-week period in winter (Heldmaier *et al.* 1982). Circulating leptin levels in Siberian hamsters are very low in winter when appetite is low, and very high when BW and fat deposition are high (Rousseau *et al.* 2002). Similar observations have been reported in sheep (Miller *et al.* 2002), woodchucks (Concannon *et al.* 2001), and root voles (Wang *et al.* 2006). This phenomenon of BW regulation by transient acquisition of leptin resistance is also observed during pregnancy in humans (Henson & Castracane 2006, Ladyman *et al.* 2009). It appears that in the seasonally breeding mammals, leptin sensitivity rather than leptin is the direct factor in seasonal changes in appetite and BW, and circulating leptin levels are directly correlated to the amount of fat stores. This means that leptin levels are high when appetite and fat stores are high, and are low when appetite and fat stores are low. In contrast, in two reports concerning non-mammalian vertebrates, leptin levels have been shown to be inversely correlated to fat accumulation with respect to the seasonal behavior of rainbow trout (Kling *et al.* 2009) and lizards (Spanovich *et al.* 2006).

Dramatic seasonal changes in BW and fat stores and the phenomenon of seasonal gonadal regression observed in some seasonally breeding mammals and in lower vertebrates are all

common to the Adélie penguins and the bar-tailed godwits. Therefore, elevation of leptin activity in the blood samples of Adélie penguin and bar-tailed godwit was expected in the high-fat seasons (courtship and refueling stages respectively) in case of similarity to mammals or possibly in the low-fat seasons (guard and arriving stages respectively) in case of similarity to rainbow trout and lizards. Our inability to detect leptin activity at any of these stages in these birds suggests that their seasonal control of appetite and fat accumulation is not mediated by circulating leptin or by leptin resistance.

The well-characterized key role of mammalian leptin in the control of energy homeostasis appears to be only partially shared with leptins in non-mammalian vertebrates (Volkoff 2006, Ronnestad *et al.* 2010). Leptin and LEPR in evolutionarily early vertebrates show relatively low sequence similarity as compared with their mammalian orthologs. For this reason, their genes were only recently identified, and currently, not much is known about their physiological roles. In some evolutionarily lower vertebrates, leptin has been implicated in the regulation of adiposity and reproduction, in addition to the regulation of developmental processes (Peyon *et al.* 2003, Crespi & Denver 2006). In addition, characterized pattern of expression, such as dominant expression in the skin and testis in salamander (Boswell *et al.* 2006) or in the liver in rainbow trout (Pfundt *et al.* 2009), suggests that the role of leptin in these animals differs from that in mammals. In experiments involving leptin treatment, no effect on food intake or BW could be detected in salmon (Baker *et al.* 2000), catfish (Silverstein & Plisetskaya 2000), or green sunfish (Londrville & Duvall 2002). Although much work is still needed to characterize the role of leptin in these vertebrates, it appears to be less tightly connected to signaling the amount of fat stores than the mammalian leptins.

Previous studies have reported PCR cloning of chicken leptin with extremely high sequence similarity to mouse leptin (Taouis *et al.* 1998). Reports on the expression of this chicken *leptin* gene at the mRNA and protein levels were also published (Ashwell *et al.* 1999a,b, 2001, Richards *et al.* 1999, 2000, Dridi *et al.* 2000b, 2005a, 2008, Kochan *et al.* 2006, Hu *et al.* 2008, Rao *et al.* 2009, Yang *et al.* 2009). This and other leptins were reported to attenuate appetite, fat metabolism, reproduction, and immune response following their application to birds (Denbow *et al.* 2000, Dridi *et al.* 2000a, 2005a,b, 2007, Benomar *et al.* 2003, Lohmus *et al.* 2003, 2004, 2006, Paczoska-Elasiewicz *et al.* 2003, 2006, Cassy *et al.* 2004, Kuo *et al.* 2005, Figueiredo *et al.* 2007, Yang & Denbow 2007), and to affect proliferation, apoptosis, and secretory activity in cultured chicken ovarian cells (Sirotkin & Grossmann 2007) and CLEPR expression in cultured chicken hepatoma cells (Cassy *et al.* 2003). In contrast, immunization against leptin decreased appetite and egg-laying (Yang *et al.* 2009); moreover, other reports showed no effect of exogenous leptin by i.c.v. injection in chicks (Bungo *et al.* 1999) and in a chicken model of skeletal bone growth (Mauro *et al.* 2010).

In addition to these controversies, accumulating evidence suggests that the *leptin* gene is missing in avian species (Friedman-Einat *et al.* 1999, Pitel *et al.* 2000, 2009, Amills *et al.* 2003, Hen *et al.* 2008, Sharp *et al.* 2008), stemming from studies based on whole genome and EST sequencing (van Hemert *et al.* 2003, Burt 2006); genomic mapping (Pitel *et al.* 2000, 2009, Liu *et al.* 2007); PCR (Friedman-Einat *et al.* 1999, Amills *et al.* 2003); library screening (our unpublished results); Southern, northern, and western analyses (Friedman-Einat *et al.* 1999, Sharp *et al.* 2008); and immunohistochemical analyses (Sharp *et al.* 2008). In addition, adipose tissue gene expression arrays have failed to detect leptin in lean or fat strains of broilers (Wang *et al.* 2007). Similarly, extensive microarray analysis of chicken fat, liver, and other tissues has failed to identify leptin (Cogburn *et al.* 2004, 2007, Wang *et al.* 2007), as has extensive EST screening of fat and other tissues (Cogburn *et al.* 2003, Carre *et al.* 2006) and proteomic analysis of abdominal adipose tissues of fat and lean broilers (Wang *et al.* 2009).

Since much of these data are based on sequence similarity, it was of primary importance to explore the possibility of circulating leptin activity in birds, using the CLEPR-based bioassay, which does not depend on sequence similarities.

One could speculate that exogenous leptins activate CLEPR, despite our inability to identify its natural ligand. This speculation is compatible with the present and previous demonstrations of CLEPR binding to heterologous leptins (Niv-Spector *et al.* 2005) and its induction by these leptins to activate signal transduction *in vitro* (Adachi *et al.* 2008, Hen *et al.* 2008). However, it is evident that more work is needed to verify the physiological role of CLEPR since the reported physiological responses were obtained with leptin doses much above the physiological concentration of leptins in mammals.

The possibility that the CLEPR may operate through an autocrine or a paracrine circuit cannot be excluded but can neither be supported until the indisputable identification of a chicken leptin.

In summary, our results strongly suggest that in birds, an alternative signaling system to that of the mammalian leptin informs the brain and other tissues of the body's fat stores. Therefore, birds may represent an excellent model system for the study of alternative control mechanisms on appetite and energy metabolism which could be shared by other non-mammalian vertebrates.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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