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Effects of chronic leptin administration on nitric oxide production and immune responsiveness of greenfinches

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ABSTRACT

Leptin and nitric oxide (NO) are both important messengers in intra- and intercellular communication systems in vertebrates. Several studies have demonstrated an involvement of both substances in the immune response. Here we tested the effects of chronic leptin and anti-leptin treatments on the NO production and phytohaemagglutinin- (PHA) induced cutaneous inflammatory response in a wild passerine, the greenfinch (*Carduelis chloris*). Plasma leptin levels of individual birds were consistent in time but could be still temporarily increased by administration of recombinant chicken leptin. Increase of plasma leptin was also induced by administration of anti-leptin, which can be most likely explained by increased endogenous leptin production due to disruption of signalling pathways. Contrary to previous findings in mammals, leptin administration reduced systemic NO production. Leptin increased cutaneous swelling response to PHA. This immune-enhancing effect was observable despite the similar plasma leptin levels of leptin-treated and control birds at the time of measurement of immune responses, i.e., 9 days after start of the treatments. This provides evidence for a delayed or long-term potentiation of the cells and cytokines involved. The effects of leptin administration on NO production and immune responsiveness were age-dependent, which indicates the complexity of underlying regulatory mechanisms.

1. Introduction

All functions in an organism depend on intra- and intercellular communication systems (Bogdan, 2001b). For many years, adipose tissue was considered to be simply a triglyceride reservoir. With the discovery of leptin and other adipocytokines, the regulatory role of adipose tissue has been established in a large number of physiological communication systems (Otero et al., 2005). Consequently, the physiological role of adipose tissue in vertebrates has been reassessed from solely being storage to an endocrine tissue of great importance. Aside from its physiological importance, the endocrine system is recently being acknowledged as a powerful mechanism behind the evolutionary adaptations of animal behaviour and ecology in complex life-history traits. Since the products of the endocrine system, hormones, often act simultaneously on many different target tissues, endocrine regulation may respond to environmental changes with coordinated evolution of entire suites of traits (Williams, 2008).

Leptin is a peptide hormone that is mainly produced by white fat-cells and released into the circulatory system (Gertler, 2006). Leptin is transported through the blood–brain barrier via a saturable transport system and most of its metabolic effects are achieved by central interactions with specific receptors in the arcuate nucleus. However, leptin receptors are also found in most peripheral tissue, facilitating direct peripheral effects of the hormone (Margetic et al., 2002; Beltowski et al., 2004b). Normally, the plasma concentration of leptin is proportional to the amount of adipose tissue in most species. An increase in the level of leptin signals the hypothalamus to activate a negative feedback loop that usually leads to a reduction in food intake and engagement in other activities (Lõhmus and Sundström, 2004; Gertler, 2006). Leptin's role as an important metabolic signal mediating many physiological functions has been documented in many vertebrate species including rodents, farm animals, humans and some domesticated and wild birds (Lõhmus et al., 2003; Gertler, 2006; Ohkubo and Adachi, 2008; Quillfeldt et al., 2009; Kordonowy et al., 2010). A decrease in leptin concentration during starvation decreases the activity of processes with high energy demand such as reproduction and immune responses (Otero et al., 2005).

Several studies have demonstrated an increase in circulating leptin during infection and inflammation, suggesting that leptin is part of the immune response and host defence mechanism (La Cava et al., 2004). Effects of leptin on T-cell activity have been documented in humans, primates, rodents, several kinds of farm animals and both wild and domesticated birds (Lõhmus et al., 2004; Otero et al., 2005; Alonso-

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Alvarez et al., 2007). Additionally, leptin synthesis has been shown to increase in response to bacterial infections and lipopolysacharide challenge, sepsis and secretion of inflammatory mediators such as IL-1, IL-6, TNF-α, and LIF (Otero et al., 2005). Leptin also acts on monocytes/macrophages by inducing the synthesis of eicosanoids, nitric oxide and pro-inflammatory cytokines. Within an evolutionary context, it is critical for organisms to divide energetic resources between processes with high energy demand without deleterious effects on survival. Therefore the role of leptin has likely evolved as a signal about the status of current energy supplies; this information may be transmitted, for example, to the hypothalamic-pituitary axis to regulate the release of reproductive hormones, or to the immune system to regulate the costs associated with health and immunity.

Another versatile messenger in the immune system is nitric oxide (Bogdan, 2001a). Nitric oxide is synthesized from L-arginine by a group of evolutionarily conserved isoenzymes. In animals NO can function as a gaseous hormone that is involved in many organismal systems, including the nervous, cardiovascular, renal, pulmonary, endocrine, and immune systems. NO has been reported to interact with several kinds of neuropeptides including NPY and leptin, and it has been proposed that the ability of leptin to reduce food intake is mediated by NO (Jang et al., 2007). The role of NO in the immune system is controversial; both protective and toxic effects have been described (Bogdan, 2001a). Overproduction of NO during an inflammatory response damages both targeted infectious organisms and healthy tissue in the vicinity. Reaction of NO with superoxide anions produces a strong oxidant, peroxynitrite, which is one of the most important initiators of oxidative damage.

Several lines of evidence suggest that high chronic leptin levels can cause cardiovascular diseases, including arterial hypertension, by activating the sympatric nervous system (Beltowski et al., 2004b). However this mechanism cannot solely explain the hypertensive effects of leptin and other possible mechanisms are likely to be involved. Oxidative stress has often been associated with reduced NO bioavailability and is known to contribute to the development of several kinds of hypertension (Beltowski et al., 2004b). Reactive oxygen species (ROS) accelerate the NO breakdown by binding with it to form peroxynitrite ($ONOO^-$) and thereby increasing the effects of oxidative stress. This ROS-mediated NO inactivation can be caused by obesity with chronic hyperleptinemia, providing a link between the actions of leptin and NO in oxidative stress.

In the present study, the effects of chronic leptin and anti-leptin treatment on NO production and immune responsiveness were investigated in a wild passerine, the greenfinch. Leptin administration in rodents increases systemic NO production (reviewed by Beltowski et al., 2002; Sweeney, 2002); however, whether this also applies to other taxa is unknown. The effects of leptin on systemic NO production have never been studied in birds, whose lipid metabolism differs from that of mammals (Ohkubo and Adachi, 2008). We tested the effects of chronic leptin and anti-leptin administration on the NO system in birds. Additionally we investigated whether leptin administration affects a phytohaemagglutinin- (PHA) induced cutaneous inflammatory response. Enhancement of PHA response by leptin administration has been documented in two studies of birds (Lõhmus et al., 2004; Alonso-Alvarez et al., 2007) but the generality of those findings awaits for further clarification. On the basis of previous findings we predicted the treatment of leptin to increase immune responsiveness to PHA. The anti-leptin was expected to act as a typical antagonist by binding to leptin receptors with an affinity similar to the non-mutant leptin, and in this way have an opposite effect to the ones observed in leptin treated birds. We also asked whether plasma leptin levels are individually consistent by measuring correlations between individual leptin levels at different stages of the experiment. Such knowledge is valuable as to carry any meaningful information about between-individual differences, physiological measures have to be repeatable in time (e.g., Hõrak and Cohen, 2010).

2. Methods

We captured male greenfinches in mist-nets in the Sõrve Bird Observatory on the island Saaremaa (57° 55′ N; 22° 03′ E) on 5–6 January and in Tartu (58° 22′ N; 26° 43′ E) on 7–8 January 2008. Birds were housed indoors in individual cages (27 × 51 × 55 cm) with sand bedding in Tartu. In the aviary, temperature and humidity averaged 15.8 ± 1.8 (SD) °C and 53.4 ± 2.9%, respectively. The birds experienced natural day length (artificial lighting) during the experiment, and were supplied with sunflower seeds and water *ad libitum*. To compensate for naturally low carotenoid content of sunflower seeds, all birds received 18 μg/mL carotenoid solution from 10 January to 1 February. Carotenoid supplementation consisted of lutein and zeaxanthin (20:1, w/w), prepared from OroGlo liquid solution of 11 g/kg xanthophyll activity (Kemin AgriFoods Europe, Herentals, Belgium).

After transportation to aviaries, birds were allowed a 7-day acclimatization period (see Fig. 1). Birds were divided into three groups (14–15 birds in each) for the leptin, anti-leptin and control (saline) treatments. These groups were selected to have similar average body mass at capture and age composition (6–8 1st-year and 7–9 older birds in each group). On the morning of 16 January, pre-experimental blood samples (200 μL) were collected. On 21 January (5 days after first blood sampling) all birds were implanted with osmotic minipumps, releasing hormone, its antagonist or vehicle. Ovine leptin antagonist (mutant L39A/D40A/F41A/I42A) cat no. LAN-5 (Protein Laboratories Rehavot) and chicken leptin cat no. LEP-2 (Protein Laboratories Rehavot) were dissolved first in one third of 20 mM Tris–HCl buffer (pH 8.9) and then in two thirds of PBS to obtain 10 mg/ml of leptin solution and 1.7 mg/ml ovine leptin antagonist solution. Alzet micro-osmotic pumps (model 1002) contained 100 μl of leptin, anti-leptin or PBS solution. Pumps released 0.25 μl of solution per hour during 14 days; thus birds received 60 μg of leptin per day or 10 μg of anti-leptin per day. Dose of leptin was derived on the basis of previous experiments with passerine birds (Lõhmus et al., 2003) and the dose of anti-leptin on the basis of Brunner et al. (1999).

The micro pump was inserted through a small incision in the ventrolateral skin. The area of incision was previously covered with a small amount of Ozonol Antibiotic Plus Ointment (SmithKline Beecham; Canada) to prevent infections and stinging. Later visual inspections revealed formation of scared tissue around the incision site but we found no evidence about inflammation (i.e., swelling or redness). On 24 January (3 days after implantation and 8 days after first bleeding) the birds were blood sampled second time.

A phytohaemagglutinin (PHA) skin test was used to measure the immune responsiveness. Subcutaneous injection of PHA induces T-cell mitogenesis and produces a localized swelling response involving local infiltration of tissue by most types of immune cells. The magnitude of this swelling can reflect both acquired T-cell-mediated immunocompetence (Tella et al., 2008) and non-specific basophile-mediated inflammation (Martin et al., 2006). Hypercellulality, caused by PHA, disappears 48 h after injection but the effects on circulating immune cell populations can last as long as for 30 days (Sarv and Hõrak, 2009). In the evening of 29 January (8 days after implantation), all birds were injected subcutaneously in the wing web with 0.2 mg of PHA (Sigma, St. Louis, MO., L-8754) in 0.04 mL of sterile isotonic saline. Wing web thickness was measured before injection and the swelling response was measured 24 h later following the simplified protocol (Smits et al., 1999) as described in detail by Saks et al. (2003). A third blood sample was collected on 30 January, i.e., in the morning following PHA injection. A fourth blood sample was collected on 20 February from a subset of birds to measure individual consistency of plasma leptin levels over time. All blood samples were collected before the lights were turned on in order to obtain the values of biochemical parameters characteristic to the state of overnight fast. Before each blood sampling, body mass of the birds was recorded with a precision of 0.1 g. Blood for determination of

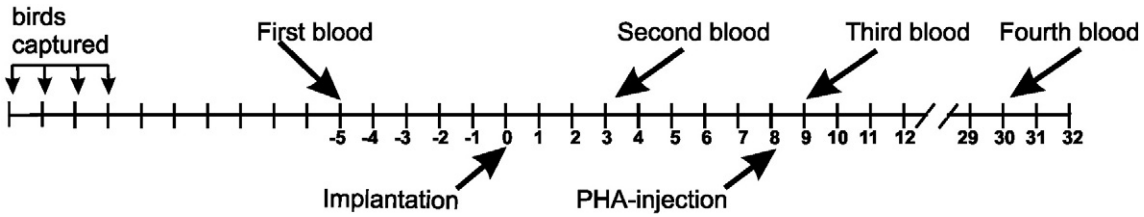

Fig. 1. Time course of the experiment. Day 0 = January 21.

plasma leptin was collected into Microvette tubes with EDTA dipotassium salt as anti-coagulant. Osmotic minipumps were removed on 1st February and the birds were released in Tartu on 3rd March. The study was conducted under a license from the Estonian Ministry of the Environment and complies with EC Directive 86/609/EEC for animal experiments. The safety of our manipulations for birds was evidenced by recapture of one of the leptin-implanted greenfinches in February 2009 in Sõrve.

Leptin samples where measured using a 1-plex mouse gut hormone panel for leptin (MGT-78 K, LINCOplex Kit). NO production was quantified from 10 μL plasma samples on the basis of measuring stable end-products of its oxidation—the nitrate and nitrite concentrations, as described in detail by Sild and Hõrak (2009). The principle of the assay is reduction of nitrate to nitrite by copper-coated cadmium granules, followed by colour development with Griess reagent. Because NO production was assessed on the basis of summed concentrations of NO_3^- and NO_2^- in the plasma, measurement units are expressed as μM NO_x for the sake of brevity. Repeatability (Lessells and Boag, 1987) of NO_x levels was 0.94 ($F_{7,25} = 59.1$, $P < 0.0001$).

Effects of experimental treatments on the dynamics of plasma leptin, NO_x levels and body mass were analyzed by repeated measures analyses of variance (ANOVA). Effect of treatments on PHA-induced swelling response was measured in an ANOVA. All models were tested for the effects of the age of the birds (yearling vs older). Assumptions for parametric tests were met for all variables, for the plasma leptin concentrations this required ln-transformation. All tests are two-tailed with an α-level below 0.05 as a criterion for significance.

3. Results

Plasma leptin levels of individual greenfinches, measured before the treatments were started, did not correlate significantly with leptin levels measured 8 days later, i.e., three days after the implantations ($r = 0.23$, $P = 0.141$, $n = 43$). However, plasma leptin levels of individual birds measured on day 3 correlated significantly with these measured on day 9 ($r = 0.42$, $P = 0.005$, $n = 44$). In the end of the experiment, we also found a significant correlation over 21 days, i.e. between the values measured on day 9 and day 30 ($r = 0.51$, $P = 0.011$, $n = 24$; all correlations with ln-transformed data).

Yearling greenfinches had significantly higher plasma leptin levels than older birds (420 ± 1 (SD) pg/mL, $n = 18$ vs 249 ± 1 (SD), $n = 24$ pg/mL; back-transformed from the ln-transformed values of least square means calculated from the model in Table 1A). When both leptin and anti-leptin treatments were included in the repeated measures ANOVA model, adjusting for age, there was a general effect of treatments on plasma leptin levels (Table 1A, Fig. 2), while time treatment interaction term was not significant. This was evidently because leptin levels in the anti-leptin group were systematically higher than among controls (Fig. 2). When the model was re-run excluding the birds from the anti-leptin group, the time treatment interaction term become significant (Table 1B), thus indicating that leptin implantation resulted in temporarily increased plasma leptin levels in greenfinches.

Treatments had no effect on dynamics of body mass ($F_{4,84} = 0.1$, $P = 0.797$ for the time treatment interaction term in repeated measures ANOVA). However, there was a significant negative correlation between the change in leptin level and the change in body mass between the pre-implantation period and day 3 after implantation (Fig. 3). Leptin implantation led to temporary decrease in plasma NO_x levels (Table 2, Fig. 4). Significant age effect in the model was caused by the stronger decline of plasma NO_x in older birds as compared to yearlings. Leptin treatment significantly increased PHA-induced wing-web swelling in old but not among yearling birds (Table 3, Fig. 5). Anti-leptin treatment had no effect on PHA-response (Fig. 5).

4. Discussion

4.1. Plasma leptin levels

Plasma concentrations of leptin were significantly elevated in leptin-supplemented birds after three days of chronic treatment, but did not differ from those of control finches nine days after the surgery. In several studies involving chronic leptin treatment, subjects appear to become resistant to exogenous leptin treatment after a certain period of time (Harris et al., 1998; Martin et al., 2000; Lõhmus et al., 2006). The present experiment showed a similar pattern; nearly all treatment effects appeared three days after implantation while all the treatment groups became similar by day nine after implantation (Fig. 2). Martin et al. (2000) suggested a down-regulation of leptin receptor mRNA and protein in the hypothalamus as a possible mechanism for the observed leptin resistance. However, as the increased plasma levels of leptin also declined after nine days of treatment, it seems more likely that chronic supplementation with external leptin in greenfinches activated a negative feedback loop that reduced endogenous leptin production. Because the effects of anti-leptin were still present at the day nine, it is not likely that the osmotic pumps had at this point extracted their entire content.

The prolonged effect of the anti-leptin treatment, specifically the increased plasma leptin concentrations, was unexpected. A possible

Table 1
The effects of leptin, anti-leptin and saline treatments on plasma leptin levels of captive greenfinches in a repeated measures model adjusting for age. In all tables "Age" means yearling vs older birds and "Time" represents the within-individual repeated measure (from 5 days before implantation to 3 and 9 days after implantation). The direction of effects and sample sizes for treatment groups can be tracked from the figures.

Effect	df	*F*	*P*
A. Model with leptin, anti-leptin and saline treatments			
Univariate tests			
Age	1,38	4.96	0.032
Treatment	2,38	3.44	0.042
Multivariate tests			
Time	2,76	16.74	<0.0001
Time × Age	2,76	0.28	0.758
Time × Treatment	4,76	1.52	0.206
B. Model with leptin and saline treatments only			
Univariate tests			
Age	1,24	6.39	0.018
Treatment	1,24	2.19	0.152
Multivariate tests			
Time	2,48	11.91	<0.0001
Time × Age	2,48	0.56	0.577
Time × Treatment	2,48	3.65	0.034

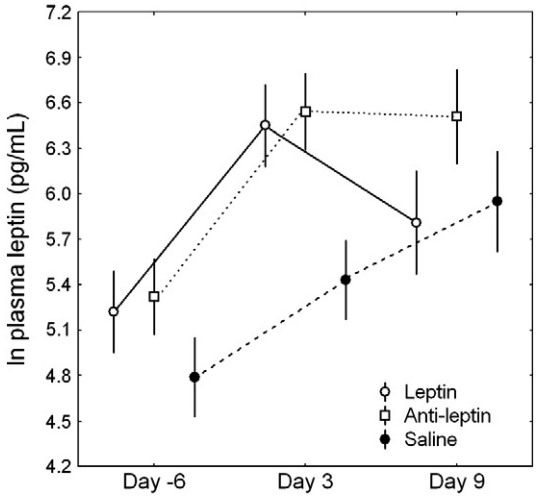

Fig. 2. Dynamics of plasma leptin levels in greenfinches in relation to the time of implantation (day 0). Least square means ± SE from the model in Table 1A. N = 13 for leptin, 15 for anti-leptin and 14 for saline treatments.

explanation could be that, because the central leptin receptors were occupied by anti-leptin molecules, the central system reacted to the decrease in leptin signalling by up-regulating the production of endogenous leptin, resulting in several fold higher plasma leptin concentrations than were observed in the control birds.

Significant correlations between individual leptin levels over six and 21 days during the second half of the experiment indicate that at least under some conditions, plasma leptin levels are individually consistent. To our knowledge, this finding is a first documentation of individual consistency of leptin levels in birds. This means that plasma leptin in greenfinches can be considered as a relatively stable biomarker of individual condition, which reflects persistent differences between the birds. Such differences may reflect genetically or developmentally determined variation in individual patterns of resource allocation.

4.2. Leptin and body mass

A comparison between leptin, control and anti-leptin treatment groups of wild greenfinches did not reveal any significant treatment effects on body mass. However, individuals with the greatest increase in plasma leptin concentration lost more weight than birds whose plasma leptin increased less (Fig. 3). The latter observation is in accordance with the 'classic' findings of previous leptin studies in mammals (Ahima and Osei, 2004) but contradicts some recent findings in wild birds (Quillfeldt et al., 2009; Kordonowy et al., 2010). On the other hand, several studies in domestic chickens (reviewed by Quillfeldt et al., 2009) have shown that administration of recombinant (chicken or human) leptin at pharmacological doses reduced food intake; the same was also observed in wild-caught great tits (*Parus major*) (Lõhmus et al., 2003). Altogether, the published evidence suggests that the links between plasma leptin and body mass dynamics cannot be always easily established, particularly in wild birds whose body mass varies usually much less than that of captive or domesticated animals (Kordonowy et al., 2010).

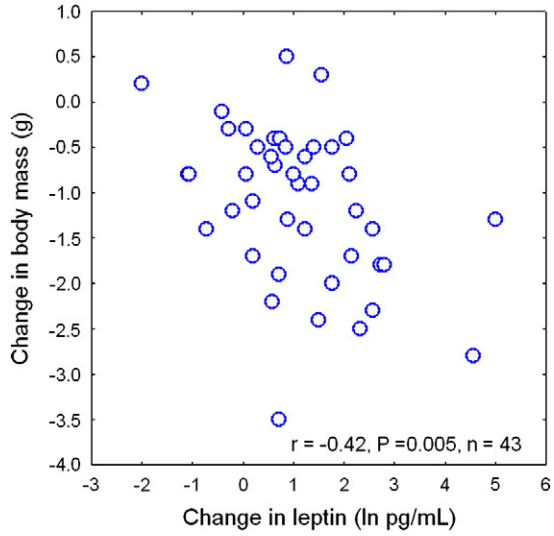

Fig. 3. Correlation between changes in plasma leptin and body mass during the period of 6 days before and 3 days after implantation.

Table 2
The effects of leptin, anti-leptin and saline treatments on plasma NO_x levels of captive greenfinches in a repeated measures model adjusting for age.

Effect	df	*F*	*P*
Univariate tests			
Age	1,30	3.74	0.063
Treatment	2,30	0.35	0.709
Multivariate tests			
Time	2,60	9.30	0.0003
Time × Age	2,60	5.72	0.005
Time × Treatment	4,60	2.70	0.039

4.3. Leptin and nitric oxide production

Chronic treatment with leptin significantly decreased the systemic levels of NO_x in greenfinches. This finding is the opposite to mammalian studies showing an increase in NO production as a response to leptin treatment (reviewed by Sweeney, 2002; Beltowski et al., 2009). This result once more illustrates the differences in leptin function between birds and mammals (Quillfeldt et al., 2009; Kordonowy et al., 2010). It is thus possible that different physiological mechanisms affect the leptin–NO relationship in birds than in mammals. For instance, in birds the liver, rather than white adipose tissue is the primary organ of lipogenesis (see Lõhmus et al., 2004) and NO production by avian heterophils may be lower than that of mammalian neutrophils (Harmon, 1998; He et al., 2008). Leptin administration has been shown to reduce NO production in chicken brain (Yang and Denbow, 2007); however, plasma leptin levels are

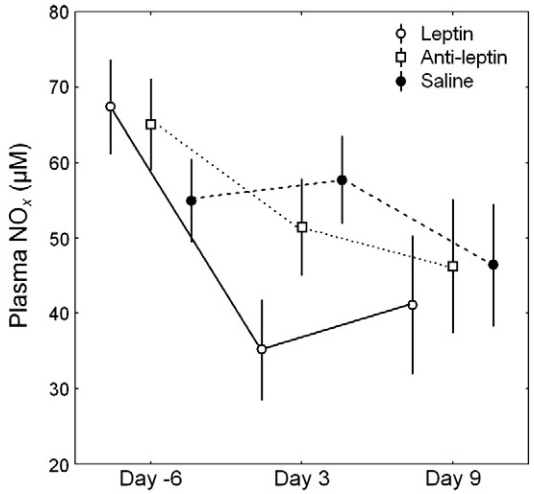

Fig. 4. Dynamics of plasma NO_x levels in greenfinches in relation to the time of implantation (day 0). Least square means ± SE from the model in Table 2. N = 10 for leptin, 11 for anti-leptin and 13 for saline treatments.

Table 3
The effects of leptin, anti-leptin and saline treatments on Phytohaemagglutinin-induced wing-web swelling.

Effect	df	*F*	*P*
Age	2,38	0.56	0.457
Treatment	2,38	7.62	0.002
Age × Treatment	2,38	6.31	0.004

more likely affected by inducible NO synthetase than neuronal NOS activated in brain (e.g., Chapman and Wideman, 2006).

Other studies have indicated that increased leptin levels can enhance oxidative stress by causing impaired NO bioactivity (Beltowski et al., 2004a). Leptin has been shown to stimulate mitochondrial oxidation of fatty acids leading to an increased production of reactive oxygen species (ROS). Additionally, leptin's immune-stimulatory role can lead to increased production of ROS by inflammatory cells such as TNF and interleukin-6 that are well-known stimulators of the superoxide generation (Beltowski et al., 2004b; Konukoglu et al., 2004; Yang and Denbow, 2007). ROS accelerate NO breakdown by binding with it to form peroxynitrite ($ONOO^-$) and in that way increase oxidative stress. Unfortunately our data do not enable a clear indication of whether the leptin treatment actually led to overproduction of reactive species which would eventually lead to reduced systemic NO production. In any case, it is possible that the propensity of leptin to decrease the NO production is one possible trade-off leading to stabilising selection of leptin levels in birds. If so, we would expect the patterns of interactions between leptin, immune response and oxidative stress to differ with the life-histories of species.

4.4. Age-dependent effects of leptin on phytohaemagglutinin response

Besides being classically considered as a hormone, leptin is designated as a cytokine as well. The structure of the leptin molecule and its receptor indicate that leptin is a member of the type I cytokine family that is characterized by a long-chain four-helix bundle (Faggioni et al., 2001; La Cava et al., 2004; Otero et al., 2005). Cytokines have the capability to regulate responses to infections and inflammatory stimuli. When a cytokine cascade is initiated it causes physiological changes such as hypoglycaemia, induction of acute-phase response proteins and anorexia. Similar to cytokines, leptin levels are greatly increased during infection and inflammation and leptin acts in a manner similar to the cytokine response to inflammation or infection, exerting direct effects on T lymphocyte proliferation, macrophage phagocytosis and secretion of inflammatory cytokines (Gabay and Kushner, 1999; La Cava et al., 2004). In accordance with this, we found a strong stimulating effect of leptin on T-cell-mediated swelling response in old greenfinches. Similar effects of leptin on T cell-mediated immunity has been demonstrated in a number of studies and in several kinds of organisms (Lord et al., 1998; Faggioni et al., 2000; La Cava et al., 2004; Lõhmus et al., 2004; Otero et al., 2005; Alonso-Alvarez et al., 2007). However, in the present study we showed that the immune-enhancing effect of leptin was observable even when the leptin levels of the leptin-treated group had dropped to the level of saline-treated birds. This provides evidence for a delayed or long-term potentiation of the cells and cytokines involved.

Another exceptional finding of our study was that leptin enhanced swelling responses only in old but not among yearling birds. Similarly, depression of NO production by leptin was stronger in older birds than in yearlings. Very few previous studies have discussed or analysed age effects in leptin physiology, making our findings novel. A couple of studies have observed that young or fast growing organisms do not react to exogenous leptin treatment with a reduction in weight or appetite (Proulx et al., 2002; Cassy et al., 2004), indicating fundamental differences in leptin biology between young and adult individuals. One possible interpretation of these patterns would be that the interactions between different physiological mechanisms become more sensitive to disturbances with an increasing age, which indeed has been suggested as a mechanism for ageing (e.g., Cichon et al., 2003). Alternatively, yearling birds might present a phenotypically or genetically different cohort of individuals because they have been exposed to selection for a shorter period (e.g., having not been tested for the ability to survive the first winter). Such an explanation would imply very strong natural selection upon the regulation of inflammatory responses by leptin. Given the massive energetic and immunopathological costs of inflammatory responses (e.g., Sorci and Faivre, 2009), such a strong selection pressure on mechanisms regulating inflammation is perhaps to be expected.

5. Conclusions

Our study showed that plasma leptin levels in greenfinches are individually consistent but yet respond to exogenous administration recombinant chicken leptin. Increase of plasma leptin was also induced by administration of anti-leptin, a result most likely explained by

Fig. 5. Phytohaemagglutinin-induced wing-web swelling in relation to the birds' age and treatment. PHA was injected 8 days after implantation and swelling measured 24 h after injection. Bars denote mean ± SE; dots are individual observations. Statistics are given in Table 3.

increased endogenous leptin production due to disruption of leptin signalling pathways. Leptin administration reduced systemic nitric oxide production, contrary to that of previous findings in mammals. Leptin increased T-cell mediated cutaneous swelling response to phytohaemagglutinin. Both of the above-mentioned effects were different in yearling and older birds. It is thus evident that leptin interferes with the nitric oxide system and immune responses. The age dependence of these effects indicates the complexity of underlying regulatory processes and possibly also strong natural selection on the regulation of inflammatory processes by leptin. Altogether, these findings highlight the value of extending the studies of the functions of leptin from traditional mammal models to passerine birds.

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