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Effect of *in ovo* Leptin Administration on the Development of Japanese Quail

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Summary

Potential changes in the activity of endocrine axes related to growth as a result of leptin administration during embryonic development of birds were evaluated in the Japanese quail as a model bird with fast growth and development. On day 5 of incubation, 0.1 μg or 1 μg of recombinant mice leptin in 50 μl of phosphate buffered saline were injected into the albumen of eggs. Animals from each group were killed by decapitation on day 0, 2, 5, 7, 14, 21, 28, 35, 42, 49 and 56 of life. Plasma concentrations of triiodothyronine (T_3), thyroxin (T_4), corticosterone, testosterone, total lipids, triacylglycerols, cholesterol, glucose and alkaline phosphatase activity were measured. Quail treated by leptin hatched earlier (5-24 hours) and had a higher body weight than the control group ($P<0.05$-0.001). Mean body weight across the whole observed period was higher in both treated groups as compared to the control group ($P<0.05$). Leptin *in ovo* administration was accompanied by changes of endocrine and metabolic parameters during postembryonic development. The most prominent changes appeared immediately after hatching (T_3, T_4, total lipids, triacylglycerols) and before sexual maturity. It is suggested that leptin acts as a general signal of low energy status to neuroendocrine systems in birds which improves utilization of nutrients.

Key words

Leptin • Japanese quail • *In ovo* manipulation • Postembryonic development • Hormones

Introduction

Hormone leptin and its receptors are key regulators in the control of food intake, growth and reproduction as well as in fat metabolism, glucose metabolism, energy expenditure and puberty in mammals (Campfield *et al.* 1996, Tartaglia 1997, Friedman and Halaas 1998, Morrison *et al*. 2001). It is widely believed that the primary physiological role of leptin is to prevent obesity by regulating food intake and thermogenesis through actions on hypothalamic centers (Tartaglia *et al*. 1995). It was shown that the effects of leptin are mediated through leptin receptors which are found not only in the brain but also in many other tissues including the lungs, kidneys, ovaries, liver and skeletal muscles. The sensitivity of the brain to leptin appears to be related to the animal's genetic background. The gene encoding leptin in chickens has recently been cloned (Taouis *et al*.

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1998, Ashwell *et al*. 1999). The gene is expressed not only in adipose tissue but also in the liver, in contrast to the mammals. Both mentioned tissues are believed to be peripheral sites involved in food intake regulation in birds (Lepkovsky 1983, Lacy *et al*. 1986).

There is increasing evidence that leptin has systemic effects apart from those related to energy homeostasis, including regulation of neuroendocrine and immune functions and a role in development. Leptin may already play an important role during embryonic development, as evidenced by production of leptin in the placenta as well as by widespread expression of leptin and its receptors in fetal tissues of mammals (Masuzaki *et al*. 1997, Hoggard *et al*. 1997). Moreover, the presence of leptin mRNA was proven in the brain, bursa, heart, liver, muscles and the spleen of 5-day-old chick embryos (Ashwell *et al*. 1999). Leptin expression was already detectable in 72-hour-old developing chick embryos (McMurtry *et al*. 2000), suggesting early leptin synthesis in avian embryos.

There are different opinions on the homology of mouse and chicken leptin (Taouis *et al*. 1998, Friedman-Einat *et al.* 1999, Dridi *et al.* 2000, Doyon *et al.* 2001). Since it was found that chick embryonic muscle cells and hepatocytes are sensitive to mouse leptin and that leptin effects on the proliferation and protein synthesis *in vitro* were dose-dependent and related to the age of embryos from which the cells were isolated (Lamošová and Zeman 2001), we used commercially available mouse leptin in our *in vivo* experiments. The possibility of changing endocrine activity related to growth as a result of leptin administration during embryonic development of birds was the aim of our study. As a model bird we used the Japanese quail which exhibits fast growth and development. On the basis of the above mentioned results we injected leptin into quail eggs on day 5 of incubation.

Methods

Animals

Eggs of the Japanese quail from our breeding colony were divided into three groups (93 in each group, with the same mean weight: 9.2±0.25 g) and incubated in a forced draught incubator with automatic turning every two hours at a temperature 37.5±0.2 °C and relative moisture 50-60 %. On day 5 of incubation, 0.1 μg (L_1) or 1 μg (L_2) of recombinant mice leptin (Pepro Tech, EC, England) in 50 μl of phosphate buffered saline (PBS) were injected into the albumen of eggs and 50 μl of PBS in the control group. Before manipulation the eggs were disinfected and holes in the eggs after injection were closed by wax. The mortality of embryos and hatchability were recorded. Birds were kept in boxes by groups up to 35 days of life, then they were moved into individual cages. The ambient temperature in the breeding room was 37 °C for the first 2 days and then decreased stepwise by 3 °C at 4-day intervals to reach 21 °C. Continuous lighting was imposed throughout the experiment. The feeding mash (21 % crude protein, 12 MJ/kg of metabolizable energy) and water were available *ad libitum*. Individual body weight and food consumption (per group till 35 days of life and then individually) were recorded daily. Six animals from each group were killed by decapitation on day 0 (day of hatching), 2, 5, 7, 14, 21, 28, 35, 42, 49 and 56 of life. Blood samples were collected into heparinized tubes. Plasma was stored at –20 °C until assayed for hormones and metabolic parameters. Parts of the duodenum and jejunum were taken for measuring alkaline phosphatase activity and a part of liver for determination of total lipids. Samples of the duodenum and jejunum were frozen and stored at –20 °C. Sections 7 μm thick were prepared in a cryostat at –20 °C, the tissue slices were transferred to glass slides and air-dried. From each tissue segment six sections were cut for enzyme assay.

Methods

Concentrations of thyroxin (Biogema, Slovakia), triiodothyronine and testosterone (Immunotech, Slovakia) in the plasma were measured by direct radio-immunoassays (RIA). Corticosterone concentrations were determined by RIA as described by Ježová *et al*. (1994). Plasma concentrations of glucose, total cholesterol, triacylglycerols and activity of alkaline phosphatase were assayed by commercially available kits (Roche, Switzerland) using the Hitachi 911 analyzer (Roche, Switzerland). Total lipids in the plasma and liver were measured by Bio-la-test (Lachema, Brno, Czech Republic).

Determination of alkaline phosphatase activity in the duodenum and jejunum was performed using the modified simultaneous azo-coupling method (Lojda 1979). The incubation medium contained: naphthol AS-BI phosphate (Sigma, Deisenhofen, Germany), fast blue BB (Aldrich, Germany), N,N-dimethylformamide (solvent for naphthol AS-BI phosphate) and veronal acetate buffer (pH 9.2). The samples were incubated at 37 °C for 10 min, using substrate concentration of 2.0 mmol/l and pH 8.9. Enzyme activity was cyto-photometrically analyzed with a Wickers M85

microdensitometer (USA). The measurements were performed at x 40 objective magnification, an effective scanning area of 28.3 μm^2 and a scanning spot of 0.5 μm at a wavelength of 480 nm. The integrated absorbance was measured in at least 30 brush border areas along the villus length (from the cryptal parts to the tip) in the duodenal and jejunal sections. Alkaline phosphatase activity was evaluated as the absorbance values recorded by the instrument/min/μm^3 brush border ± S.E.M.

All data are presented as means ± S.E.M. The results were analyzed by two-way analysis of variance (ANOVA). Comparisons within the single sampling days were made by the Student's *t*-test. Body weight data were fitted by Gompertz function (Starck and Ricklefs 1998). Data were analyzed using SigmaPlot and SigmaStat software.

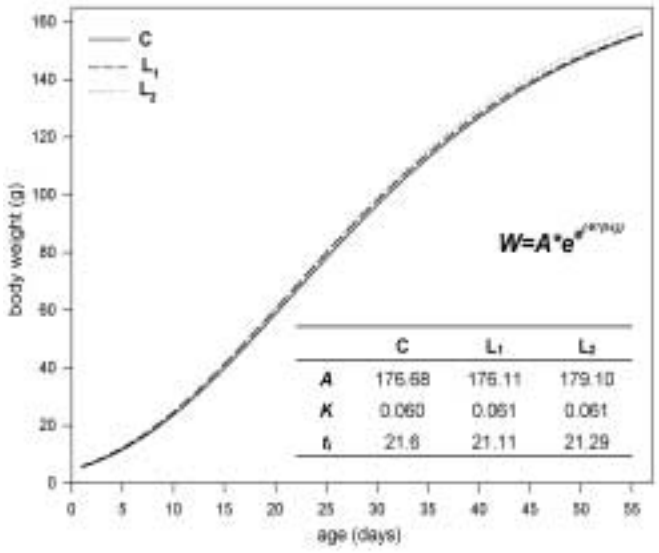

Fig. 1. *Japanese quail growth curves fitted by Gompertz equation after in ovo leptin administration on day 5 of incubation. Inserted table: estimated equation parameters of distinct treatment groups. A – asymptotic body mass, K – growth rate constant, t_i – inflection point of the growth curve; C - control group – solid line; L_1 - 0.1 μg of leptin – dashed line ; L_2 - 1μg of leptin – dotted line.*

Results

There were no differences in fertility of eggs among groups (C=77.3 %, L_1=76.8 %, L_2=77.7 %) and hatchability of fertilized eggs was at the same level (71.20±0.36 %). No developmental aberrations were observed during incubation and at hatching. Quail treated during embryonic period by leptin hatched earlier than the control group in four independent experiments: birds injected with 1 μg of leptin by 8-24 hours and with 0.1 μg of leptin by 5-12 hours. The mean body weights of hatched quail were as follows: control (C): 6.23±0.068 g; treated with 0.1 μg of leptin (L_1): 6.69±0.073 g ($P<0.001$ versus C) and 1 μg of leptin (L_2): 6.46±0.067 g ($P<0.05$ versus C). Sex ratio, found at autopsies, was as follows: C: 53.1 % of males : 46.9 % of females, L_1: 54.8 % of males : 45.2 % of females, L_2: 55.4 % of males : 44.6 % of females.

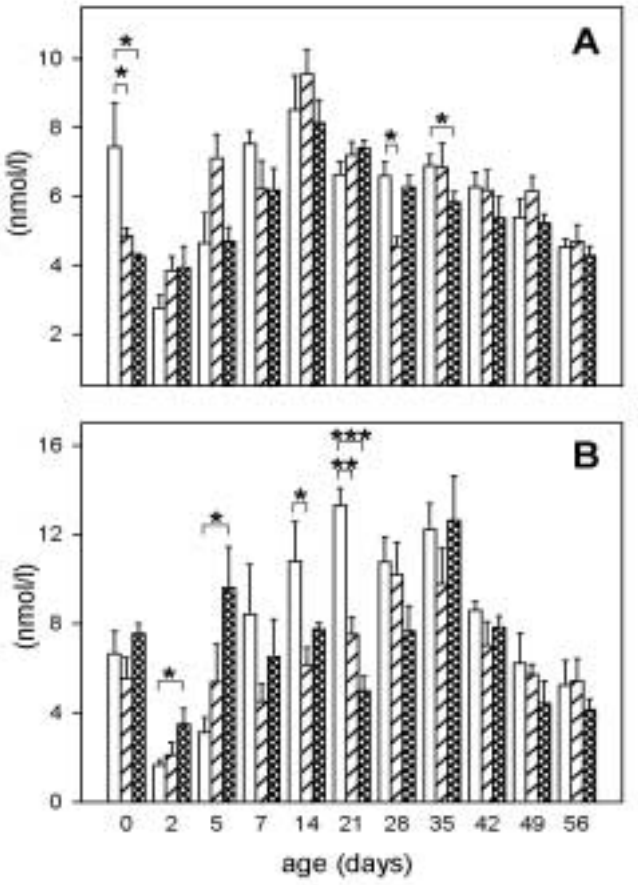

Fig. 2. *Plasma concentrations of thyroid hormones during postembryonic development of Japanese quail after in ovo leptin administration on day 5 of incubation.* ***A*** *-* T_3 *,* ***B*** *-* T_4 *. Data are means from six birds ± S.E.M. C – control groups – open columns;* L_1 *- 0.1 μg of leptin – hatched columns;* L_2 *- 1 μg of leptin – cross-hatched columns. * P<0.05; ** P<0.01; *** P<0.001 vs control.*

There was a significant effect of treatment ($F_{2,2960}$=16.6, $P<0.001$) and age ($F_{32,2960}$=5391.6,

P<0.001) on body weight. Mean body weight across the whole observed period was higher in both treated groups as compared to the control group (*P*<0.05) (Fig.1).

Leptin injected into eggs during embryonic period affected thyroid hormone (TH) levels in plasma (Fig. 2 A, B). T_3 levels were significantly lower (P<0.05) in hatchlings of L_1 and L_2 groups in comparison with the controls (Fig. 2A). The concentrations of plasma T_3 on the following days did not differ from the control ones. During the period of sexual maturation (28-35 days of life) concentrations of T_3 in leptin groups were again lower in comparison with the controls. Plasma T_4 levels were higher in groups injected with a higher dose of leptin than in the controls on day 2 and 5, while an opposite trend was recorded during the phase of rapid growth. Decreased T_4 concentrations in leptin-treated birds were found on day 14 and 21 (Fig. 2B).

Differences in the age of sexual maturity were found in treated and control birds. Quail treated by leptin showed a trend to a higher weight of the testes from day 35 of life (Table 1) in males and earlier onset of egg laying in females (L_1 –2 days) compared with the control group. Changes in testes weight were reflected by higher plasma testosterone concentrations in leptin-treated groups on day 49 of life (P<0.05) (Fig. 3A). Females from leptin groups (L_1, L_2) reached sexual maturity earlier (100 % of egg laying females) than the controls (Fig.3B).

Table 1. Weight of testes (g) of Japanese quail after *in ovo* leptin administration on day 5 of incubation. C – control group; L_1 – 0.1 μg of leptin; L_2 – 1 μg of leptin.

Day of life	**21**	**28**	**35**	**42**	**49**
C	0.08±0.01	0.36±0.07	1.03±0.20	2.49±0.05	3.74±0.04
L_1	0.05±0.02	0.39±0.05	1.41±0.37	2.65±0.45	3.96±0.02
L_2	0.04±0.02	0.30±0.08	1.35±0.14	2.82±0.24	3.93±0.02

Data are means ± S.E.M.; n = 6

The highest daily body weight gain was recorded in the L_1 group. A higher food intake was responsible for the higher body weight gain. Numerically the highest food intake was found in the group treated with the lower leptin dose (L_1) (Table 2).

The activity of alkaline phosphatase in the intestine did not change substantially during postnatal development (Table 2). We found a highly significant increase of alkaline phosphate activity in the duodenum (P<0.05-P<0.001) as well as the jejunum (P<0.01-P<0.001) in the group treated with a higher dose of leptin (L_2) during the whole studied period (Table 2).

Plasma corticosterone levels were gradually decreasing and reached the lowest value before a period of sexual maturation in all groups (Table 2). Birds with the higher dose of administered leptin showed a trend to higher plasma corticosterone concentration.

The effect of leptin on lipid metabolism was evaluated on the basis of total lipids in the plasma and the liver, concentrations of triacylglycerols, cholesterol and the activity of alkaline phosphatase in plasma. A significant decrease in plasma total lipids was found on hatching in the leptin groups. Later, during the period of intensive growth, concentrations of total lipids were increased in leptin groups, but they decreased again in comparison with control group before sexual maturation (Fig. 4A). A developmental pattern of total lipids in the liver exhibited higher values after hatching, decreased levels during the period of rapid growth and an expected subsequent increase which paralleled the egg laying rate (Fig. 4B). Total lipids in the liver of treated birds did not differ in comparison to the controls except day 5 (L_2 P<0.05) (Fig. 4B).

Concentrations of triacylglycerols in the plasma rose from the day of hatching to maturity in all groups (Fig. 5A). Significant differences in comparison with the controls were observed in the group with lower leptin L_1 concentration. During intensive growth, triacylglycerol levels were increased, while they were decreased before sex maturity similarly as total lipids in the plasma. The concentration of plasma cholesterol in the leptin groups did not show significant differences in comparison with the control. During egg laying, the levels of cholesterol were raised in all groups (data not shown).

Likewise, the concentration of glucose in the plasma did not show significant differences between groups and the level of glucose was in the same range during the whole experimental period (data not shown).

The activity of plasma alkaline phosphatase changed during the postembryonic period: the highest values were found during intensive growth and the highest food intake, then gradually decreased and the lowest values were achieved in sexually mature birds (Fig. 5B).

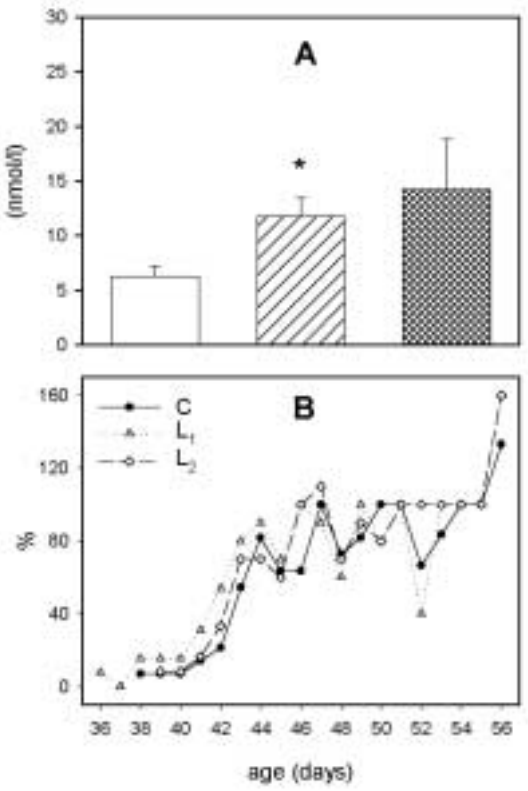

Fig. 3. *Plasma testosterone concentrations (**A**) and egg laying (**B**) of Japanese quail after in ovo leptin administration on day 5 of incubation. Data (**A**) are means from six animals ± S.E.M. Legend as in Fig. 2. * P<0.05 vs control. Data (**B**) are means from 6-14 animals ± S.E.M.*

Discussion

Manipulation of hormonal environment of the avian embryo can lead to subsequent changes in growth and development by altering the ontogeny of hormone producing cells. The alteration is realized *via* endocrine axes and the cellular response to hormones *via* changes the receptor number and their sensitivity (Hargis 1991). It was found that peripheral administration of leptin to fasting mice reverses fasting-induced changes of the adrenal, thyroid and gonadal axes (Ahima *et al*. 1996, Chehab *et al.* 1997, Ahima and Flier 2000). Our results indicate that leptin injected early in ontogeny affected embryonic development and, to some extent, also postembryonic development. Leptin treatment advanced the development of embryos because we found earlier hatching and higher body weight of birds in leptin groups in all experiments (data presented here and unpublished data). Concomitantly, the activity of the hypothalamo-pituitary-thyroid gland axis was probably advanced by the treatment and a peak of T_3 related to the pipping process occurred earlier than in controls. It is well known that concentrations of serum T_3 in the Japanese quail is the highest during pipping through the shell and then rapidly decrease till the day of hatching (McNabb 1987). Significantly lower concentrations of T_3 in leptin groups on day of hatching (< 24 h old) found in our experiments are probably related to advanced embryonic development and earlier hatching of quail from the leptin groups. Our data stress the important role of thyroid hormones (TH) in processes of development and hatching (McNabb 1995, Flier *et al.* 2000, Seoane *et al*. 2000).

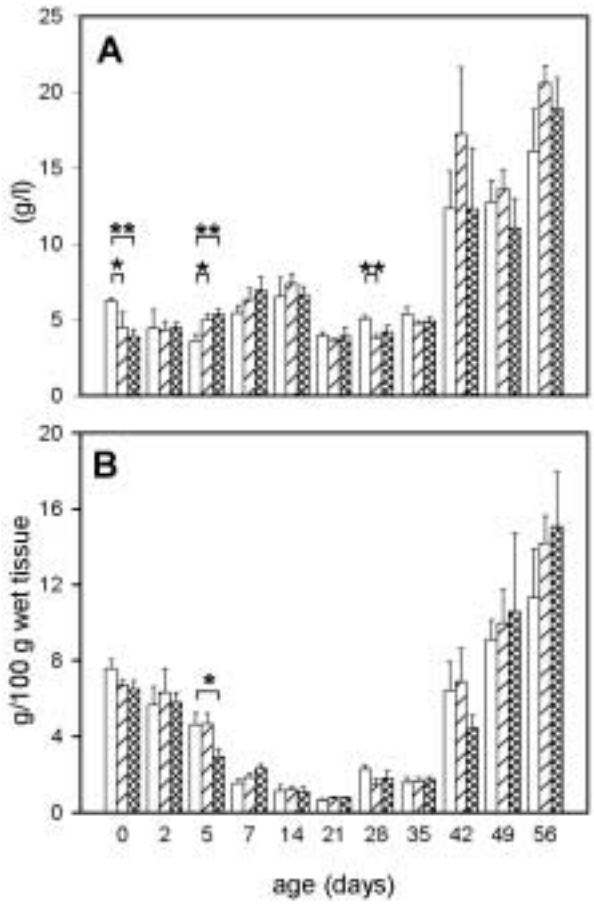

Fig. 4. *The content of total lipids in plasma (**A**) and in liver (**B**) of Japanese quail during postembryonic development after in ovo leptin administration on day 5 of incubation. Data are means from six birds ± S.E.M. Legend as in Fig. 2. * P<0.05; ** P<0.01 vs control.*

Changes of TH concentrations measured during postembryonic development in quail treated embryonically with leptin are less consistent. It is known that the hypothalamus-pituitary-thyroid axis is markedly influenced by the metabolic status (Seoane *et al*. 2000) and TH levels are major physiological regulatory factors during transition from the fed to the starved state not only in rodents (Ahima *et al*. 1996, Flier *et al*. 2000) but also in birds (Decuypere and Kuhn 1988). It is suggested that leptin also signals the switch from the fed to the starved state (Ahima *et al*. 1996, Flier and Maratos-Flier 1998). The dominant and perhaps a sufficient signal to the brain that suppresses TRH expression in the paraventricular nucleus is a drop in the level of the hormone leptin. Alterations in thyroid status and in leptin levels result in changes in body weight and energy metabolism (Wauters *et al.* 2000). In our study, decreased T_4 levels found in leptin-treated groups during the rapid growth phase before sexual maturity were not accompanied by increased T_3 concentrations. More data on the conversion of T_4 to metabolically more active T_3 or inactive reverse T_3 (rT_3) are needed to consider if thyroid hormones play a role in more intensive growth of quail treated embryonically with leptin.

Table 2. Daily food intake per one quail, activity of alkaline phosphatase in duodenum and jejunum and plasma corticosterone concentrations during postembryonic development of Japanese quail after *in ovo* leptin administration on day 5 of incubation. C – control group; L_1 – 0.1 μg of leptin ; L_2 – 1 μg of leptin.

Age (days)	Group	Daily food intake (g)	Alkaline phosphatase duodenum (μkat/l)	Alkaline phosphatase jejunum (μkat/l)	Corticosterone (nmol/l)
5	*C*	2.25	4.26±0.14	4.24±0.19	4.28±0.23
	L_1	2.49	4.44±0.15	4.56±0.31	6.16±0.52
	L_2	2.62	6.62±0.28***	6.14±0.21***	6.99±1.33
7	*C*	5.16	4.36±0.17	3.92±0.10	4.22±0.26
	L_1	5.86	4.34±0.18	4.18±0.20	4.71±0.87
	L_2	5.59	5.72±0.06***	5.34±0.15***	4.10±0.29
14	*C*	7.07	4.36±0.15	4.10±0.07	3.87±0.43
	L_1	7.46	4.16±0.18	3.96±0.11	3.29±0.20
	L_2	7.46	5.46±0.15***	4.94±0.12***	5.72±1.45
21	*C*	10.56	4.42±0.16	4.24±0.14	3.47±0.81
	L_1	10.60	4.52±0.20	4.42±0.19	3.32±0.26
	L_2	10.59	5.58±0.08***	5.35±0.17**	3.47±0.20
28	*C*	13.86	4.94±0.27	4.40±0.17	1.10±0.23
	L_1	14.53	4.36±0.12	4.30±0.17	0.92±0.26
	L_2	13.88	6.00±0.38*	6.00±0.23***	1.16±0.40
35	*C*	15.38	5.44±0.15	4.42±0.04	2.31±0.66
	L_1	16.90	4.90±0.18	4.50±0.23	4.10±1.33
	L_2	15.76	6.54±0.05***	5.50±0.14***	5.43±0.66
42	*C*	17.88±0.99	5.23±0.14	4.48±0.10	5.55±1.68
	L_1	21.13±0.92	5.06±0.14	4.64±0.21	5.14±2.08
	L_2	19.00±0.60	6.46±0.17***	5.64±0.16***	11.79±6.36
49	*C*	21.73±2.13			3.58±0.66
	L_1	25.50±1.15			2.49±0.81
	L_2	22.40±0.83			2.77±0.43
56	*C*	22.00±1.18	5.42±0.13	4.41±0.11	3.12±0.43
	L_1	25.40±2.36	5.36±0.19	4.64±0.18	3.87±0.23
	L_2	25.40±1.75	6.58±0.19***	5.60±0.23***	4.39±0.72

*Data are means from at least six birds ± S.E.M. * P< 0.05; ** P<0.01; *** P<0.001 vs C*

The interrelationship between leptin and hypothalamo-pituitary-adrenal (HPA) axis covers the actions of glucocorticoids on leptin synthesis as well as the regulation of the HPA axis by leptin. In mammals it is documented that leptin has both stimulatory and inhibitory effects on activity of the HPA axis (Ahima *et al*. 1996, Schwartz *et al*. 1996, Bornstein *et al*. 1997, Heiman *et al*. 1997). In our study, treatment with leptin resulted in higher corticosterone levels, however, the changes were not very intensive. In both control and leptin groups, corticosterone levels exerted significant changes during postembryonic development.

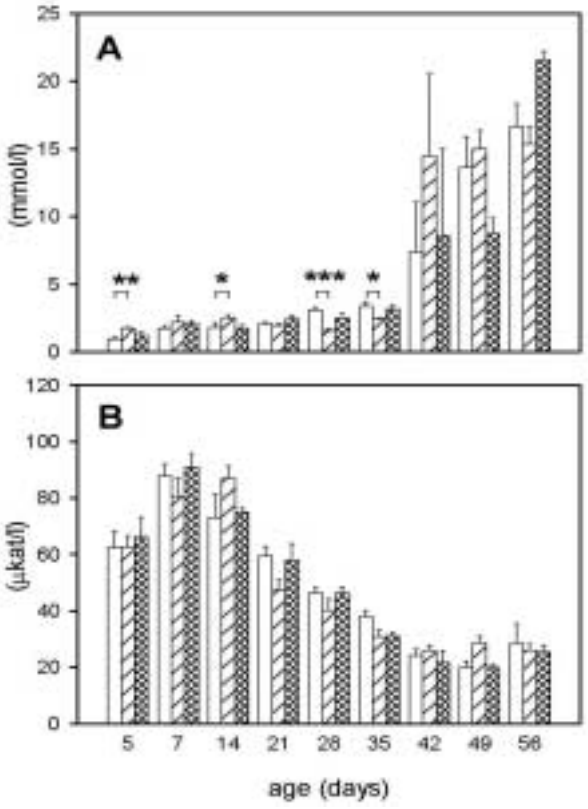

Fig. 5. *Concentrations of triacylglycerols (**A**) and activity of alkaline phosphatase (**B**) in plasma of Japanese quail during postembryonic development after in ovo leptin administration on day 5 of incubation. Data are means from six birds ± S.E.M. Legend as in Fig. 2. * P<0.05; ** P< 0.01; *** P< 0.001 vs control.*

In addition to the endocrine system, leptin treatment may influence the digestive system through the synthesis and secretion of digestive enzymes. Our data show consistent changes in the activity of alkaline phosphatase in leptin-treated birds. The stimulation of this enzyme was more pronounced in L_2 groups. In birds there is no information available on alkaline phosphatase which is a membrane-bound enzyme present in absorptive cells of intestinal villi where it may be related to nutrients, particularly lipids. Our data suggest that the development of the digestive system may be advanced by leptin treatment and it may represent a long-term advantage for treated birds. The activity of alkaline phosphatase in the duodenum was found to be higher than in the jejunum. The higher activity of this enzyme may be connected with better utilization of nutrients, resulting in higher body weight. It is interesting that the alkaline phosphatase activity in the intestine did not change from hatching to maturity in all animals. The concentration of alkaline phosphatase in the plasma (which comes from different sites of the body) decreased significantly from the period of intensive growth to maturity. Leptin administration in embryonic period did not evoke the changes in the concentration of alkaline phosphatase in plasma during the experimental period.

It has been proposed that leptin is one of signals controlling sexual maturation in mammals and it is also involved in the regulation of reproductive functions. Leptin effects on sexual maturity are mediated *via* receptors in hypothalamic neurons that influence GnRH neuron activity. However, in birds, there is a lack of data about leptin effects on reproduction. In all our experiments, female quail from both leptin groups reached sexual maturity earlier than control birds. We suppose that higher leptin levels in embryonic development induced faster development of embryos (earlier hatching, higher body weight at hatching) and evoked earlier onset of sexual maturity.

In mammals, a number of *in vitro* experiments showed that leptin has a direct autocrine or paracrine mode of action on the rate of synthesis and degradation of lipids. There is a basic physiological difference between birds and mammals in lipid biosynthesis. In birds, a substantial part of lipid is synthesized in the liver (Saadoun and Leclercq 1983) and this fact is also reflected in the expression of leptin mRNA in this organ (Ashwell *et al*. 1999). We found significant differences between leptin and control birds in plasma triacylglycerols (TG) and total lipid levels during postnatal development. Higher TG levels in plasma of leptin-treated quail after hatching can reflect increased degradation of lipids from depots by leptin. The total

lipids in plasma expressed a similar pattern as TG. On the other hand, total lipids in the liver of leptin-treated quail had decreased concentrations during the first days of postnatal development. This finding supports a possibility of higher lipid degradation at the beginning of postnatal development in birds treated by leptin during embryonic development.

Leptin has been shown to impair many aspects of insulin effects on glucose metabolism including stimulation of glucose transport and glycogen synthase activity in rodents (Muller *et al*. 1997). *In vivo* glucose utilization by rat brown adipose tissue actually increased with leptin treatment (Siegrist-Kaiser *et al*. 1997). The concentration of plasma glucose of our leptin groups did not differ from the control group during postnatal development.

Leptin administration into eggs of the Japanese quail accelerated embryonic development and resulted in higher body weight of hatched quail. Stimulatory effects of injected leptin were also recorded after hatching. Birds in both leptin-treated groups hatched earlier with higher body weight and expressed a higher growth rate. This finding suggests that treatment with leptin during the embryonic period resulted in permanent changes of control mechanisms regulating growth and development. It is hypothesized that leptin in birds acts as a general signal of low energy status to neuroendocrine systems that improve the utilization of nutrients.

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