# Embryonic and Post-Embryonic Development of Japanese Quail after *In Ovo* Melatonin Treatment

Z. KOVÁČIKOVÁ[1], T. KUNCOVÁ[1], D. LAMOŠOVÁ[2], M. ZEMAN[1,2],

[1]Comenius University, Department of Animal Physiology and Ethology, Bratislava, Slovakia
[2]Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Ivanka pri Dunaji, Slovakia

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**Abstract**

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The effect of exogenous melatonin on embryonic and post-embryonic development of quail was analysed. Japanese quail (*Coturnix coturnix japonica*) eggs were incubated under standard conditions and light regime L12:D12. At incubation day 5 eggs were treated with 5, 50 or 250 μg of melatonin per egg and at incubation day 10 with 50 or 250 μg melatonin/egg. Melatonin was injected into eggs in 50 μl of sterile saline containing maximum 5% of ethanol. Fertility and hatchability of eggs were recorded. Hatchlings were inspected for gross developmental aberrations and killed by decapitation during post-embryonic day 1, 2 and 3 in the middle of the scotophase and photophase. Plasma and pineal glands were collected and melatonin levels were measured in both materials by radioimmunoassay. Weights of the liver, spleen and bursa of Fabricius were recorded at postembryonic day 1, 2 and 3. Plasma melatonin levels were increased in melatonin treated groups in comparison with control group at day 1 and 2. High hormone levels persisted until post-hatching day 2. These findings suggest that melatonin was not metabolized efficiently during the embryonic period and embryos were under melatonin influence during embryonic development. Administration of melatonin did not affect pineal melatonin levels. No developmental malformations were found in treated birds. Hatchability and weights of organs did not differ between the melatonin - treated and control groups.

*Melatonin, quail, development, embryo*

Melatonin, produced by the pineal gland, is involved in the control of circadian rhythms. Its production has a strictly circadian pattern and is driven by an endogenous circadian clock. Concentrations of this hormone are high during the dark-time and low during the light-time. Melatonin is released into blood stream immediately after synthesis, and therefore its plasma levels reflect production in the pineal gland (Binkley 1988).

Circadian production of melatonin in mammals starts after birth (Kaufman and Menaker 1991; Yellon et al. 1985; Mirmiran et al. 1992). In contrast to mammals, in birds the rhythmic pattern of melatonin production is seen during embryonic development (ED). In chickens, rhythmic production was confirmed at day 18 of ED under *in vivo* conditions (Zeman et al. 1992) and the amplitude of the rhythm was very low. At day 20 of ED the amplitude was 10-fold higher than at day 18. A subsequent increase of melatonin content is observed between embryonic day 19 and post-embryonic day 3 (Herichová et al. 2001). Circadian production of melatonin under *in vitro* conditions was confirmed at day 14 of ED (Akasaka et al. 1995) and at day 16 (Lamošová et al. 1995). Rhythmic melatonin synthesis was observed also in embryos of another precocial bird species, the Japanese quail (Zeman and Gwinner 1992). This early production of melatonin in birds in comparison with mammals may reflect an adaptation to the absence of maternal melatonin influencing mammalian fetus (Zeman et al. 1999).

**Address for correspondence:**
Zuzana Kováčiková
Department of Animal Physiology and Ethology
Comenius University, Mlynská dolina B–2
842 15 Bratislava, Slovakia

Phone: 00 421 2 602 96 424
Fax: 00 421 2 654 29 064
E-mail: mzeman@fns.uniba.sk
http://www.vfu.cz/acta-vet/actavet.htm

Melatonin is generally considered a non-toxic compound (Sugden 1983; Weaver 1997). Melatonin does not affect the development of mouse embryos (McElhinny et al. 1996) under *in vitro* conditions but very high melatonin concentrations have an embryotoxic effect (Chan and Ng 1995). Under *in vivo* conditions no deleterious effects of melatonin were recorded (Chan and Ng 1995).

Generally there are limited data about effects of melatonin on development. There is no information about an influence of this hormone on ED of birds although avian embryo is a well suitable model to address questions of developmental effects of melatonin. Therefore, the aim of the present study was to investigate whether exogenous melatonin injected into fertilized eggs of Japanese quail influences the pre- and post-incubation development of birds.

## Materials and Methods

Fertilized eggs of Japanese quail (*Coturnix coturnix japonica*) obtained from a breeding colony kept at the Institute of Animal Biochemistry and Genetics, SASci, Slovakia, were incubated at a temperature of 37.6 ± 0.2 ºC and relative humidity 50-60% in a forced draught incubator (Bios Midi, Sedlčany, Czech Republic). Light-dark regime 12:12 h was used. Light was provided by a fluorescent tube (Osram, Germany). Light intensity was 120-180 lux at the level of eggs. Hatched quail were kept in the incubator under the same temperature and light conditions as eggs for 48 h after hatching. When quails were studied for 3 days (see Experiment 3) they were removed from the incubator after hatching and placed in plastic cages in a brooding room with free access to water and feeding mash for young chicken. Temperature in the room was kept at 36 ± 1ºC.

Melatonin (Sigma, USA) was dissolved in a small amount of ethanol (maximum 5%) and then mixed with sterile saline to required concentration. Injected volume was 50 µl per egg. Eggs from the control group were injected only with saline containing the same proportion of ethanol as the experimental group. Egg shells were disinfected with ethanol before injection, melatonin solution was administrated into egg white and the opening was sealed by paraffin.

Hatchability of quail was recorded in each experiment and hatchlings were inspected immediately after hatching for developmental abnormalities.

Blood and pineal glands were taken from 1-, 2- and 3-day-old quail in the middle of the light and dark phase. Blood was collected after decapitation into heparinized tubes and immediately after collection it was centrifuged for 10 min at 1 500 *g*. Plasma was separated and stored at -20º C until assayed for melatonin. Concentrations of melatonin in plasma were measured directly and in the pineal gland after extraction with methanol.

Melatonin was measured by radioimmunoassay (RIA) (Fraser et al. 1983) validated for quail plasma in our laboratory (Zeman et al. 1993). In RIA [$^3$H]-labelled melatonin with a specific activity 3.056 TBq/mmol (NEN Du Pont, Germany) and sheep melatonin antiserum (G/S/704 8483 Stockgrand Ltd., University of Surrey, U.K.) were used.

Experiment 1

Eggs (n = 75) were divided into three groups (each of 25 eggs). At day 5 of ED one group was treated with 5 µg of melatonin, one with 50 µg and control group with saline/ethanol solution. Plasma melatonin levels were measured in the dark phase in 1-day-old quail.

Experiment 2

Eggs (n = 78) were divided into three groups (each of 26 eggs). Eggs were treated at day 10 of incubation with 50 µg of melatonin, 250 µg of melatonin and control group with saline/ethanol solution. Plasma melatonin levels were measured in the dark phase in 1-day old hatchlings.

Experiment 3

Melatonin (250 µg) was administered at day 5 of ED into 118 eggs and other 103 eggs were treated with vehicle only. After hatching quail were inspected for developmental abnormalities. Melatonin levels were measured in plasma and in the pineal glands, which were collected in the middle of the light and dark phase in 1-, 2- and 3-day old quail. Weights of the spleen, bursa of Fabricius and liver were recorded in both groups at all three days.

Data were analyzed by multifactorial ANOVA test with post-hoc Tukey´s test.

## Results

Administration of 5 µg melatonin/egg at day 5 of ED did not affect plasma melatonin levels in 1-day-old quail and plasma melatonin levels were in same range in both the control and melatonin treated group. The dose of 50 µg/egg injected at the same day of ED significantly ($p < 0.05$) increased levels of plasma melatonin in 1-day-old quail in comparison with control and 5 µg of melatonin/egg group (Fig. 1).

Treatment with 50 µg of melatonin per egg at day 10 of ED did not influence levels of

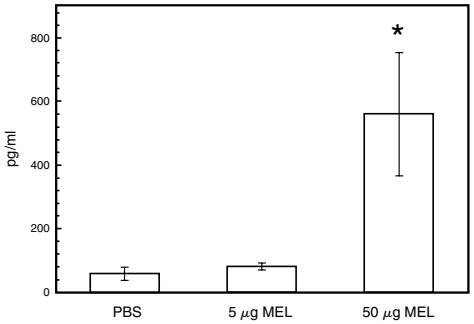



Fig. 1: Plasma melatonin concentrations in 1-day-old Japanese quail after melatonin injection on embryonic day 5. Values represent means ± SEM, * $p < 0.05$

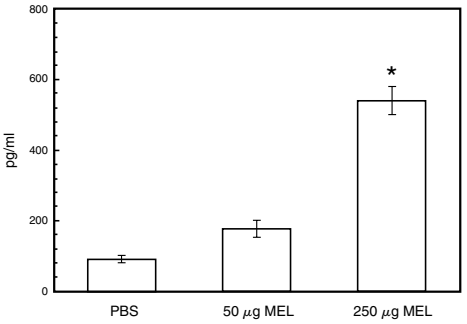


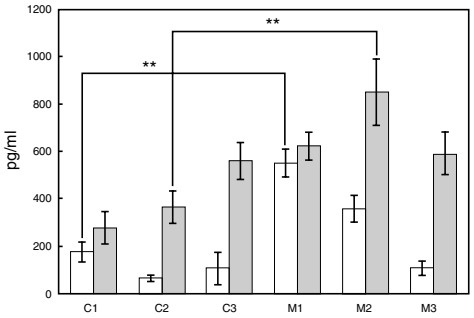

Fig 2: Plasma melatonin concentrations in 1-day-old Japanese quail after melatonin injection on embryonic day 10. Values represent means ± SEM, * $p < 0.05$

circulating melatonin at post-embryonic day 1. Administration of 250 μg of melatonin significantly ($p < 0.05$) increased plasma melatonin concentration in comparison with the control and 50 m g of melatonin/egg group (Fig. 2).

Injection of 250 m g melatonin per egg at day 5 of ED increased plasma melatonin levels during postnatal day 1 and 2 in comparison with the control group (Fig. 3). A significant difference was observed between the control and the experimental group in the middle of the light time at post-hatched day 1 ($p < 0.01$) and between the control and experimental group in the middle of the dark time in 2-day old quail ($p < 0.01$). Differences between the control and experimental group in the mid-dark phase of day 1 and in the mid-light of day 2 were not significant but there was a tendency for higher levels in the experimental group. At day 3 there were no differences between the control and experimental group (Fig. 3).

Administration of 250 m g of melatonin at embryonic day 5 did not affect the content of melatonin in the pineal gland. No significant differences were observed between the control and experimental groups (Fig. 4).

No developmental abnormalities were found in hatchlings treated with melatonin. We observed no influence of melatonin administration on weights of the liver, bursa of Fabricius and spleen (data are not shown). Hatchability was in the same range in both experimental and control groups and administration of melatonin did not influence this parameter.




FFig. 3: Plasma melatonin concentrations in 1, 2, and 3-day- old Japanese quail. C- control group, M - melatonin treated group, 1, 2, 3 – post-hatiching day, open bars - photophase, patterned bars - scotophase. Values represent means ± SEM, ** $p < 0.01$

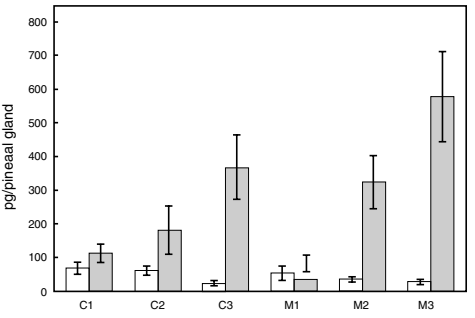



Fig. 4: Pineal melatonin content in 1, 2, and 3- day-old Japanese quail. C – control group , M – melatonin treated group, 1, 2, 3 – post-hatching day, open bars - photophase, patterned bars - scotophase. Values represent means ± SEM

## Discussion

Our data showed that exogenous melatonin administered into Japanese quail eggs during the first and the second part of embryonic development was effectively absorbed into a developing embryo. High plasma concentrations were found after hatching – e.g. 12 and 7 days after the treatment, respectively. These results were not expected because melatonin is a highly reactive compound with biological half-time - above 12 min (Vakkuri et al. 1985).

Treatment with melatonin *in ovo* induced a time dependent increase of plasma melatonin levels in hatched, 1-day-old Japanese quail. At day 10 of ED the dose of 50 μg of melatonin/egg did not exert a significant effect on plasma melatonin levels and the increase of plasma melatonin levels after application of 250 μg of melatonin/egg at embryonic day 10 was smaller (267.9±24.8 pg/ml) then after treatment with 50 μg of melatonin on incubation day 5 (558.2±191.9 pg/ml). These results can be explained by the fact that melatonin as a highly lipophilic molecule (Reiter 1993) easily penetrates into the yolk sac through biological membranes. It is possible to assume that the uptake of melatonin is lower in older embryos because their yolk sac is partially absorbed.

Finding of high plasma melatonin levels in 2 days old quail during the light and dark phase suggests that enzymatic degradation systems are not fully developed during embryonic and early post-embryonic life. It can be assumed that degradation systems are fully developed early after hatching, because plasma melatonin concentrations in melatonin treated group returned to levels comparable with the control ones at post-embryonic day 3.

The content of melatonin in the pineal gland did not differ between the control and experimental groups. It means that treatment with melatonin did not affect production of this hormone by the pineal gland. This finding corresponds with the fact that the main zeitgeber of melatonin production is a light/dark cycle and others signals (such a feedback loop) only marginally influence pineal melatonin production.

We did not find any malformations in hatchlings and no significant differences in weights of spleen, bursa of Fabricius and liver were observed between the experimental and control groups. Our results support earlier published data that melatonin is a non-toxic compound under *in vivo* conditions and it has no obvious negative effects on the development of animals (Chan and Ng 1995; Mc Elhinny et al. 1996).

A supposed role of melatonin during a prenatal period is to communicate information about environmental cycles to physiological and behavioural systems (Davis 1997). The other possibility is that melatonin couples the central circadian system with peripheral organs and supports development of their function. The first information that exogenous melatonin can alter the function of peripheral organs during ED was published by Höchel and Nichelmann (2001). Melatonin administered in physiological doses influenced heart rate of chicks and Muscovy ducks. The authors found that the first response of heart rate to melatonin coincided with the start of rhythmic melatonin production in the avian embryo. Those data as well as present results support a hypothesis that development of melatonin rhythmicity in the avian embryo may subsequently influence other rhythms in physiology and biochemistry that develop during the perinatal period.

## Embryonálny a postembryonálny vývin prepelíc japonských po aplikácii melatonínu in ovo

Sledovali sme vplyv exogénneho melatonínu na embryonálny a post-embryonálny vývin prepelíc japonských. Násadové vajcia prepelice japonskej (*Coturnix coturnix japonica*) sme inkubovali za štandardných podmienok, svetelný režim bol L12:D12. V piatom dni inkubácie sme do vajec injikovali melatonín v dávke 5, 50 alebo 250 μg/vajce, alebo v 10.

dni inkubácie v dávke 50 alebo 250 m g/vajce. Melatonín sme aplikovali v 50 m l sterilného fyziologického roztoku obsahujúcom max. 5% etanolu. Zaznamenávali sme oplodnenosť vajec a liahnivosť, ako aj výskyt vývinových malformácií. Vyliahnuté prepelice sme dekapitovali v 1., 2., a 3. dni po vyliahnutí v strede svetlej a tmavej fázy a v odobratých epifýzach a plazme sme stanovili melatonín rádioimunoanalytickou metódou. Zisťovali sme aj hmotnosti pečene, sleziny a Fabriciovej bursy. Hladiny plazmatického melatonínu boli zvýšené v skupinách, ktorým bol aplikovaný melatonín v porovnaní s kontrolnými skupinami. Toto zvýšenie pretrvalo do 2. dňa po vyliahnutí. Tieto výsledky naznačujú, že melatonín nie je počas embryonálneho vývinu metabolizovaný a môže ovplyvňovať embryo. Podanie melatonínu nemalo vplyv na množstvo melatonínu v epifýzach. Nepozorovali sme výskyt vývinových malformácií v experimentálnej skupine. Nezistili sme rozdiely v hmotnostiach orgánov medzi kontrolnými a experimentálnymi skupinami, ani rozdiely v liahnivosti vajec.

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