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Insect hormones in vertebrates: anabolic effects of 20-hydroxyecdysone in Japanese quail

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Abstract. Ecdysteroids are hormones controlling cell proliferation, growth and the developmental cycles of insects and other invertebrates[1]. They are occasionally present in various unrelated plants for no apparent reason; no phytohormonal function has yet been identified. In certain cases, ecdysteroids are accumulated to high levels in leaves, roots or seeds. Some ecdysteroid-containing plants have been known as medicinal plants for centuries. One of them, *Leuzea carthamoides* Iljin (Asteraceae), growing in Central Asia, contains 0.4% ecdysteroid in dry roots and 2% in seeds. A pharmacological preparation from this plant, 'Ecdisten', is already available as a commercial preparation for its anabolic, tonic and other effects, for medical use (review[2]). It remained problematic, however, whether ecdysteroids were truly responsible for these effects, because *Leuzea* contains a number of other biologically active compounds in addition to ecdysteroids. We extracted and purified ecdysteroids from the seeds of *Leuzea*. With 6 g of 96% 20-hydroxyecdysone (20E), we made a large-scale feeding assay with Japanese quail to find out whether ecdysteroid alone could duplicate the anabolic effects of the seeds. We found that the 96% ecdysteroid increased the mass of the developing quails in a dose-dependent manner, with the rate of increase proportional to the ecdysteroid content in the seeds; there was a 115% increase in living mass with 100 mg kg^{-1} of pure 20E compared with 109.5% increase with 100–180 mg kg^{-1} 20E equivalents in the seeds. We conclude that the plethora of growth-promoting, vitamin-like effects induced in vertebrates by *Leuzea* is mediated by ecdysteroids.
Key words. Ecdysteroids; ecdysone; 20-hydroxyecdysone; polyhydroxylated sterols; anabolic effects; *Coturnix japonica* (Aves); *Leuzea carthamoides* (Asteraceae).

The polyhydroxylated 6-keto, 7-Δ sterols – ecdysteroids (ES) – were discovered 30 years ago while screening for insect moulting hormone activity[3]. In contrast to most other sterols, which are strongly lipophilic, ES are partly soluble in water due to the presence of five or more hydroxylic groups. The original biochemical definition of the first structurally identified ES, ecdysone and 20-hydroxyecdysone (20E), proposed that these hormones were secreted by prothoracic glands for regulation of moults and developmental cycles in insects. This definition of ES survived only a few months, because phytochemists quickly uncovered a widespread occurrence of these 'insect hormones' in various taxonomically unrelated plants: phytoecdysones or phytoecdysteroids[1]. In insects and other invertebrates, ES function as true growth hormones, directly stimulating tissue proliferation. In contrast to other hormones, manufactured only in the endocrine glands, the biosynthesis of ES is not limited to the prothoracic gland. It can proceed in most or all growing insect tissues, including the peripheral epidermal targets of the hormone[4]. Due to such endocrinological abnormalities, ES have been considered as homeostatic factors for the synchronization of growth among various tissues and organs[5]. Certain parts of the plant (roots, leaves, fruit) can occasionally accumulate ES at concentrations a million times above those usually found in insects[1,2,6]. However, in spite of this finding, a reasonable explanation for why these compounds exist or how they function in plants is as yet unknown, except for insights concerning their possible defensive role against insect herbivores[6]. It is known that ES definitely do not function as phytohormones[7].

The function of ES in vertebrates has been documented by traditional pharmacology in the context of medicinal herbs. Of these, the most significant is the thistlelike, 1-m tall, perennial *Leuzea (Rhaponticum) carthamoides* Iljin, belonging to the family Asteraceae and growing in Central Asia. For centuries this drug, known as 'the root of the deer maralu', was recognized as a rejuvenator and metabolic stimulant. During investigation of its physiological effects in mammals, it was discovered in 1977[8] that it contained a very high content of 20E: approximately 1.8% dry matter in seed and 0.4% in root. Anabolic effects produced by *Leuzea* and other plants containing ecdysteroid were reviewed first in

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1984[9]. Since 1982, an ecdysteroid extract from the root of *Leuzea* has been commercialized as a pharmacological preparation, '*Ecdisten*', in the territory of the former Soviet Union. It is recommended for use against astheno-depressive states, weakeness or chronic intoxication; it has beneficial effects on neurasthenia, neurosis, hypotension, fatigue and the recovery from infectious disease[2,6].

A recent review on the effects of ES in vertebrates[2] provides a list of very important pharmacological applications in mice and rats: stimulation of protein synthesis, changes in carbohydrate and lipid metabolism, increased cell immunity, adaptogenic effects and a general stimulation of growth which was also seen in domestic animals. Moreover, antidepressive, tonic or rejuvenating effects, improved physical condition and antisenescent properties are reported reactions of the human body[2]. Due to the general stimulatory effects on growth, metabolism and neural functions, it has been concluded that ES should be considered as a new class of sterol-based essential vitamins[2].

Until recently, the majority of ES research in vertebrates has been done on rodents and other mammals, while not much information is yet available concerning other classes of vertebrates. Certain species of birds are specialized to feed on seeds, which often contain enormous quantities of ES. To obtain more information about the possible effects of ES in birds, we performed extensive feeding trials with ES in Japanese quail (*Coturnix japonica*) in 1994[10]. The quail were chosen because of their fast development, small size, early sexual maturity and relatively small food consumption[11]. In these assays[10], we fed the quail with diets containing 0.2, 1.0 and 5.0% pulverized seeds of *Leuzea* (with a content of 1.8–2.1% 20E) for 50 days. There was a dose-dependent increase in somatic growth of the quail: (i) 0.2% addition of seeds increased the living mass to 102.8%; (ii) 1% addition caused an increase of 109.5%; and (iii) 5.0% addition caused an increase of 120.4%. The *Leuzea* seed complement produced more pronounced growth-stimulating effect than some commercially available probiotic food additives for birds (Biostrong, Ecovit)[10].

In all previously investigated dietary assays with *Leuzea* (see review[2]), the true responsibility of ES for the observed biological effects was never unambiguous, since the plant contained a number of other biologically active chemicals[12], but feeding trials with pure ES would be too expensive. To clarify this problem, we began a large-scale extraction and purification of our own 20E from a substantial amount of *Leuzea* seeds. A feeding assay with the first yield of 6 g was performed on Japanese quail, since they provided a previously well characterized model.

Materials and methods

The basis for isolation of 20E was provided by 170 kg of dry seeds of *L. carthamoides* from plantations in Velký Osek, Czech Republic. The one-year-old, dry seeds were first extracted with methanol by the pharmaceutical company Galena. The concentrated extracts were prechromatographed on aluminum oxide. Fractions containing 20E were further separated by crystallization from acetone, with final purification on preparative high-performance liquid chromatography (HPLC), using procedures common for separation of ES[13]. The final product obtained by HPLC was characterized by mass spectrometry; its biological activity was ascertained by standard bioassays on insects. The purity used in this study was 96% 20E with 4% contamination, mostly by other, structurally related and biologically active ES.

The basic assay involved 200 freshly hatched quail of the strain Faraon ($n = 160$ for controls; $n = 10$ for each experimental group). The quail were fed standard food mixture BR-1 for broilers. The 20E was incorporated into the diet in acetone with final concentrations of 20, 100 and 500 mg kg^{-1}. Breeding took place in special cages (STS Hostivice) under a 16-h light:8-h dark phase photoperiod. Statistical evaluation was based on one-way analysis of variance (ANOVA) using a multiple comparisons test. Determination of 20E content in blood serum of the quail was made according to a previously described radioimmunoassay method[10].

Results and discussion

The lowest dose (20 mg kg^{-1} = 20 ppm) of 20E in the diet induced a relatively small, statistically insignificant ($p > 0.05$) stimulation of growth (fig. 1). During the initial period of the first and second weeks, the medium dose (100 mg kg^{-1} = 100 ppm) and larger dose (500 mg kg^{-1}) induced a more pronounced stimulation of growth (see fig. 1), but the values still did not differ significantly from the controls ($p > 0.05$). By contrast, both the medium and larger doses of 20E caused pronounced anabolic effects later during weeks 3 and 4, and the differences related to controls were highly significant ($p < 0.001$). However, the most pronounced dietary effects of 20E can be clearly discerned in figure 1 during the fourth week, which is puberty, when there were 115 and 131% increases in the living mass relative to the controls. Further comparison of data presented in figure 1 with the previously described dose-response relationships obtained by feeding the seeds of *Leuzea* revealed considerable similarities. With the diet containing 1% seeds of *Leuzea* (=equivalent of 100–180 mg kg^{-1} 20E) we obtained a 109.5% increase in the living mass in 1994[10]; now with 100 mg kg^{-1} of pure 20E we obtained a 115% increase (fig. 1).

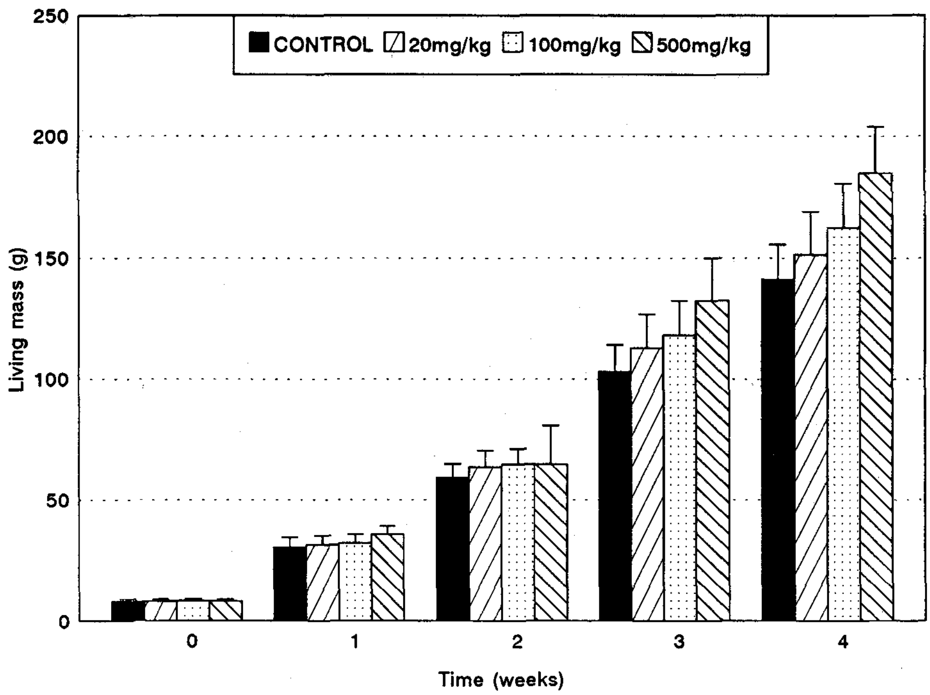

Figure 1. Dietary effects of 20-hydroxyecdysone administered in standard food mixture BR-1 for broilers (20, 100 and 500 mg kg^{-1}) in Japanese quail (*Coturnix japonica*) (bars indicate averages + SD, n = 10).

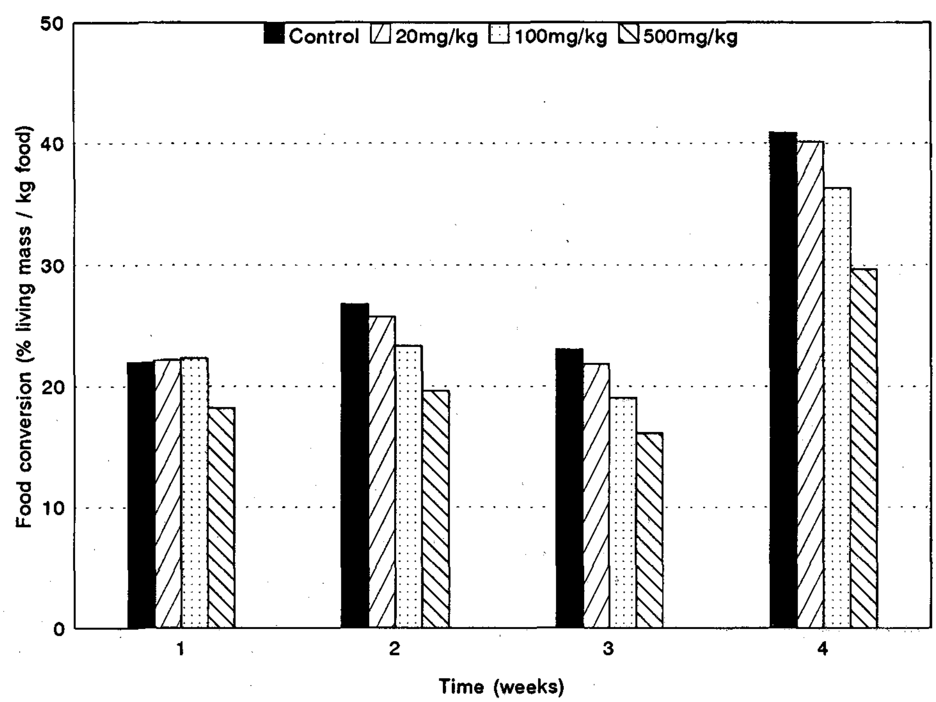

Figure 2. Values of the food conversion index during the feeding assay with 20-hydroxyecdysone in Japanese quail (for further details see fig. 1).

The similarities above provide the first experimental evidence that 20E is indeed responsible for most or all of the anabolic effects of *Leuzea* in birds, and perhaps in all vertebrates. Comparisons of the dietary effective range of ES concentration reveal that, irrespective of whether it is active as a hormone in insects or a vitamin in vertebrates, the effective range of concentrations is the same, i.e. between 20 and 100 ppm for birds and for insects[14].

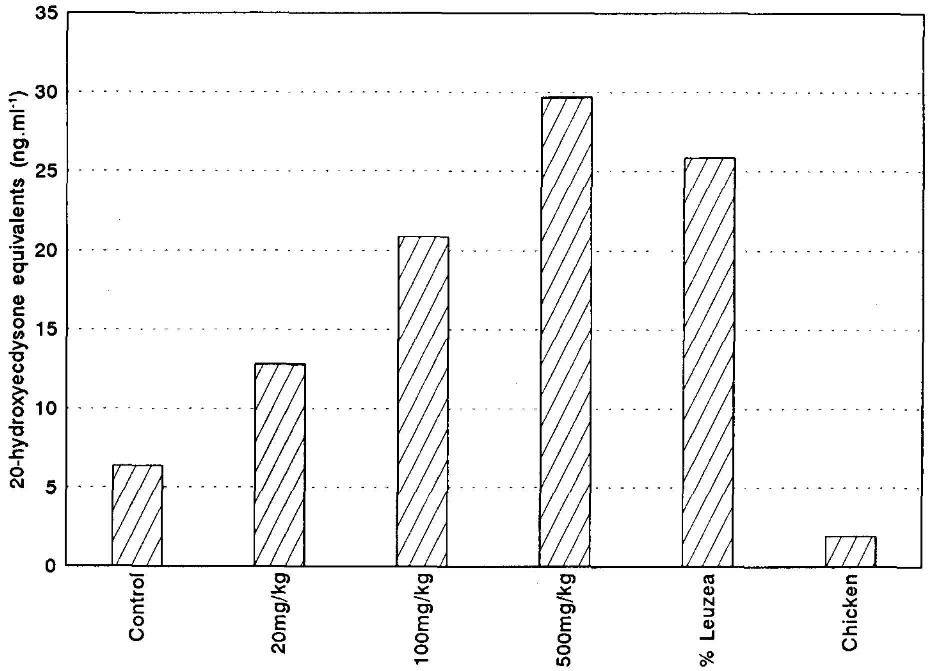

Figure 3. Radioimmunoassay determination of 20-hydroxyecdysone in blood serum of 4-week-old Japanese quail (end of the feeding assay in fig. 1). Estimated content of 20E for 1% *Leuzea* seed in the diet is 100–180 mg kg^{-1}. 'Chicken' indicates 20E in blood serum of 3-day-old chicken from F_1 generation; the parents received 500 mg kg^{-1} 20E in the diet (averages from five determinations).

Determination of the food conversion index (fig. 2) revealed that a dietary supply of 100 and 500 mg kg^{-1} of 20E substantially reduced the amount of food necessary for assimilation of a constant unit of living mass. This effect was most pronounced during the fourth week of puberty, when the quail assimilate reserve materials for future reproduction. This economy in food conversion might be the result of an enhanced rate of protein synthesis and metabolism which results from administration of ES in vertebrates[2,9]. It offers great potential for poultry breeding.

In the previous feeding assay with *Leuzea* seeds[10], we measured the titre of 20E circulating in the blood of experimental groups of the quail. The blood concentrations of 20E appeared to be directly correlated with the quantities of 20E in the diet. This finding was not fully consistent with other earlier data suggesting rapid breakdown and excretion of dietary ES within 4–10 min in mice[15] or within 8–16 min in chickens[16]. The present results obtained with pure 20E (see fig. 3) fully confirm our previous conclusion that the titers of ES in blood of quail are directly proportional to the content of 20E in food. This suggests that metabolic turnover of 20E is under physiological control in quail. It cannot be simply rapidly removed from the bloodstream. If this is true for other vertebrates as well, it would explain the elevated titers of ES in human blood[2,17] resulting from the presence of ES in food.

For a long time ES and polyhydroxylated sterols escaped the attention of organic chemists, who did not expect to find a sterol in partly water-soluble polar fractions. Moreover, pharmacologists, who are much concerned with the dietary content of cholesterol, also obstinately ignored the possibility that considerable quantities of a water-soluble, 'sugar-like', derivative of cholesterol could be ingested with vegetable food[6]. The present evidence for a true anabolic action of pure ES in vertebrates fully substantiates earlier conclusions[2,6] that the biological importance of all these slightly polar sterolic compounds may reach far beyond the limits of a specialized insect hormone. We believe that ES might indeed constitute a new class of essential sterolic vitamins that is currently ignored. The far-reaching pharmacological implications of this encourage future plans for assays with large quantities of pure ES.

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