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# THE CAPABILITY OF ANDROGEN AROMATIZATION BY BANK VOLE LEYDIG CELLS *IN VITRO:* THE EFFECT OF AROMATASE INHIBITOR

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The effect of increasing concentrations of aromatase inhibitor CGS 16949A on LH-and testosterone-supplemented estradiol secretion by Leydig cells was studied. Leydig cells were obtained from immature and mature bank voles, which were reared in a long or a short photoperiod conditions. They were either incubated for 6 hrs with LH and/or testosterone (LH/testosterone-supplemented cultures), or as the control cultures. Aromatase inhibitor was added for further 18 h. After 24-h incubation period testosterone and estradiol secretion were radioimmunologically examined.

This study revealed that in bank voles, Leydig cells' aromatase activity appeared to be photoperiod- and age-dependent. However, the differences in its activity were seen only in case of mature tissue. In immature bank voles, reared in both regimes of light aromatase activity was low, while in mature animals a 50% increase was observed. Thus, aromatase activity and estradiol biosynthesis were much lower in the Leydig cell culture from a short day than in the analogus culture fom a long one. The present study also showed different sensitivity to the stimulation of LH between young and adult bank voles kept in different regimes of light, which could be connected with the number of Leydig cells' LH receptors.

K e y w o r d s: *aromatase, aromatase inhibitor, estradiol secretion, Leydig cells, bank voles, in vitro.*

## INTRODUCTION

Aromatase cytochrome P450 (P450 arom) catalyses the aromatization process which is the conversion of androgens to estrogens. Aromatase activity has been recently studied in a great variety of tissues in most vertebrate species (1—5). However, the identity of the testicular cells that produce estrogens has been controversial. Valladares and Payne (6, 7) have suggested that in both young and adult rats only Leydig cells in the interstitial tissue showed positive

immunoreaction for aromatase, while Rommerts *et al.* (8) and Tsai-Morris *et al.* (9) have reported that the site of estrogen biosynthesis changes during development of the testis. In bank voles, which are seasonally breeding animals, photoperiod regulates growth and development of the testes and determines the beginning and the length of the breeding season. It can induce profound changes in the testes morphology and in the steroidogenic activity of Leydig cells. In the present work we show that Leydig cell aromatase activity depends on the length of the photoperiod, age of the bank voles and it is also influenced by the exogenous testosterone and LH added to the culture medium. The conversion of testosterone to estradiol by Leydig cells *in vitro* was studied using non-steroidal aromatase inhibitor CGS 16949A.

## MATERIAL AND METHODS

### *Animals*

The source of Leydig cells were testes of male bank voles from our own colony (Laboratory of Animal Endocrinology, Institute of Zoology, Jagiellonian University) reared in 6L:18D or 18L:6D conditions for 4—5 or 7—8 weeks. A standard pelleted diet and water provided *ad libitum*. Animals were killed by cervical dislocation, their testes were removed and decapsulated.

### *Cell preparation and culture*

Leydig cells, suspension for culture was obtained by digestion with 0.25% trypsin for about 10 minutes as previously described (10). Briefly, the tissue was serially sieved through 156 μm and 74 μm pore-size steel meshes (US Standard Sieve series ASTME-11 Specifications, Dual MFG, Chicago) and allowed to sediment. The supernatant was collected and centrifuged for 10 minutes at $180 \times g$. Then the supernatant was removed and the pelleted cells were resuspended in incubation solution (Medium 199 supplemented with 5% calf serum, L-glutamine and sodium bicarbonate). Penicillin (120 i.u./ml) was also added. The Leydig cells were cultured in 24-wells culture dish (Nunc, Kalmstrup, Denmark) at 37°C in humidified atmosphere of 5% $CO_2$ in air. The initial inoculum was $1 \times 10^5$ cells/ml. Cells were incubated either with $10^{-7}$ M, $10^{-8}$ M, $10^{-9}$ M of testosterone alone (Sigma Chemical Co, St. Louis, USA) or with 100 ng LH (NIH-LH, s. 20, ovine, Bethesda, USA) plus testosterone (LH/testosterone-supplemented cultures). After 6 hour of incubation three doses ((0.1, 0.5, 1.0 μg/ml) of aromatase inhibitor CGS 16949A [4-(5,6,7, 8,-tetrahydroimidazo[1,4-alpridin-5-yl]benzonitryle monochloridae (Ciba-Geigy, Basel, Switzerland) were added for further 18 hrs. Although, three different doses of testosterone were added, only influence of the highest dose was significant. The effect of the others was negligible. The purity of the cell suspension was about 85% and it was checked by histochemical test for $\Delta^5$, 3 β-hydroxysteroid dehydrogenase ($\Delta^5$, 3 β-HSD) activity. Viability of the cells assessed by trypan blue exclusion test, was $< 95\%$ (1).

### *Steroid analysis*

Samples of culture medium were analysed for androgen and estrogen content using radioimmunoassays. Androgens were determined according to Dufau *et al.* (12) using 1,2,6,7,-3H-testosterone (The Radiochemical Centre, Amersham, Buckinghamshire,

England), spec. act. 81 Ci/mmol, as a tracer and an antibody raised in rabbit against testosterone-3-o-carboxymethylo-oxime-BSA (gift from Prof. B. Cook, Glasgow, Scotland). The lower limit of sensitivity of the assay was of the order of 5 pg/tube. Cross-reactions for eight steroids were below 0.01%, but cross-reactivity of 5α-androstane-17β-ol-3-one showed 100% dihydrotestosterone (DHT) 20.8%, androstendione 15.7%, androsterone 7.4% and dehydroepiandrosterone (DHA) 3%. Coefficients of variation within and between assays were 7.5% and 9.7%, respectively.

Estrogens were assessed according to Hotchkiss *et al.* (13) using 2,4,6,7,-3H-estradiol (New England Nuclear), spec. act. 104 Ci/mmol, as a tracer, and an antibody raised in rabbit against estradiol-17β-6-oxime-BSA (gift from Prof. B. Cook, Glasgow, Scotland). The specificity of the antiserum for estrogen was high. It cross-reacted with estradiol 100%, with estrone 66% and with estriol only 2.1%. For cross-reactivity series of other steroids were tested, but they showed less than 0.1%. The lower limit of sensitivity of the assay was of the order of 5 pg/tube. Coefficients of variation within and between assays were 3.5% and 7.5%, respectively.

### *Reagents*

All reagents used were of analytical grade, purchased from the Sigma Chemical Co., St Louis, USA.

### *Statistical analysis*

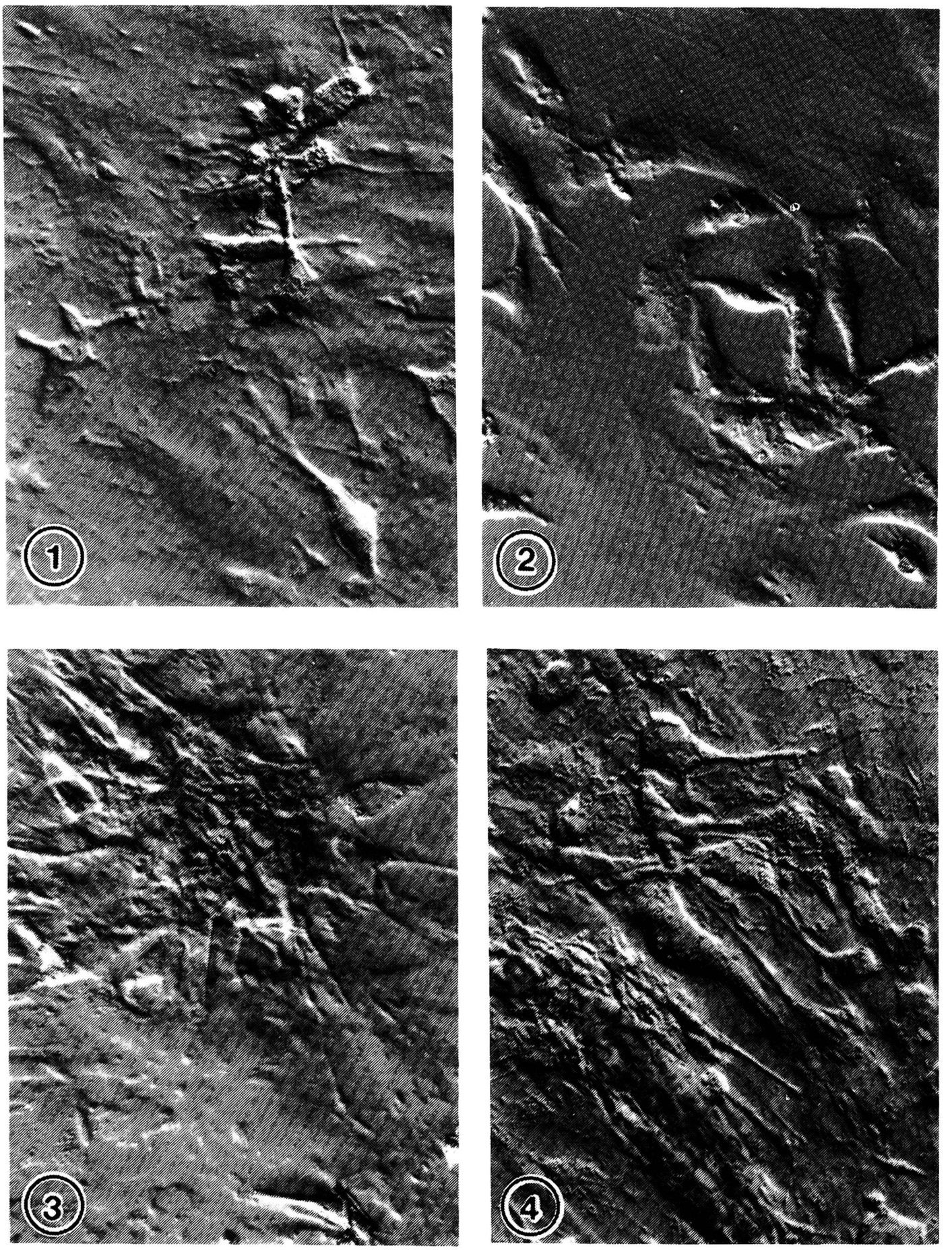
Statistical evaluation of the data included one-way analysis of variance (with the significancy at $p < 0.05$) and the Duncan's multiple range test. Means without common superscripts (a, b, c) are significantly different.

## RESULTS

### *Effect of increasing concentrations of aromatase inhibitor CGS 16949A on testosterone secretion by Leydig cells of immature and mature bank voles from a short and a long photoperiod.*

After a 24-hour-incubation, morphological analysis showed significant differences between the cultures of Leydig cells obtained from animals kept in a short and in a long photoperiod conditions. Independently of the bank voles' age Leydig cells from a long day grew as monolayer contrary to the Leydig cells from a short one growing as single cells or in clusters (*Figs 1—4*).

The basal testosterone secretion by Leydig cells of immature bank voles from a long photoperiod (4.5 ng/$10^5$ cells) was greater than from a short one (1.5 ng/$10^5$ cells). Administration of aromatase inhibitor did not affect the testosterone release independently of the dose used (*Fig. 5*).




*Figs 1—4.* 24-hour-control culture of the bank vole Leydig cells. Nomarski optics. × 200.
*Fig. 1.* Leydig cell culture obtained from immature animals kept in a short photoperiod conditions.
*Fig. 2.* Leydig cell culture obtained from mature animals kept in a short photoperiod conditions.
*Fig. 3.* Leydig cell culture obtained from immature animals kept in a long photoperiod conditions.
*Fig. 4.* Leydig cell culture obtained from mature animals kept in a long photoperiod conditions.

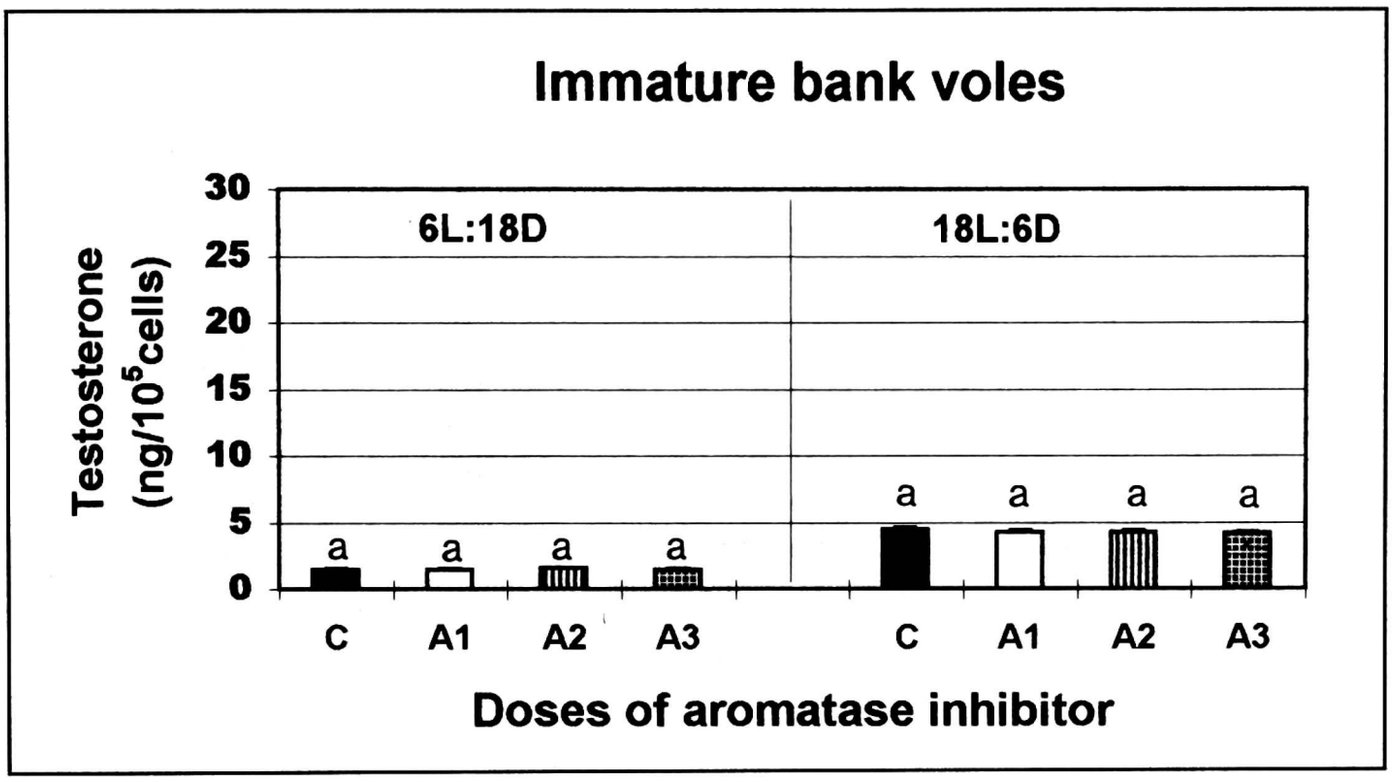



*Fig. 5.* The effect of increasing concentrations of aromatase inhibitor CGS 16949 A (A1 = 0.1 μg/ml, A2 = 0.5 μg/ml, A3 = 1.0 μg/ml) on basal testosterone secretion by Leydig cells of immature bank voles from a short and a long photoperiod. All values are mean ± SE. Means without common superscripts (a, b, c) are significantly different ($p < 0.05$).

In mature bank voles basal testosterone secretion by Leydig cells from a short photoperiod was about 3 ng/$10^5$ cells, while in a long day the testosterone release was 75% greater (14 ng/$10^5$ cells). The administration of aromatase inhibitor to the culture medium of Leydig cells from a long day resulted in a dose dependent stimulation of testosterone. In the culture of Leydig cells from a short day, only the highest dose added to the culture medium had a stimulatory effect on testosterone release (the obtained results were of statistical significance) (*Fig. 6*).

*Effect of aromatase inhibitor CGS 16949A on: a) basal, b) testosterone--supplemented and c) LH- and testosterone-supplemented estradiol secretion by Leydig cells of immature and mature bank voles from both regimes of light.*

a) The basal level of estradiol released by the Leydig cells from immature bank voles depended on the regime of light. In a short day the estradiol secretion was about 6 pg/$10^5$ cells, while in a long day it was 54% greater (13 pg/$10^5$ cells). The administration of aromatase inhibitor did not affect the estradiol secretion in a short day, while it caused a decrease in estradiol level in the culture of Leydig cells from a long one (the obtained results were of statistical importance).

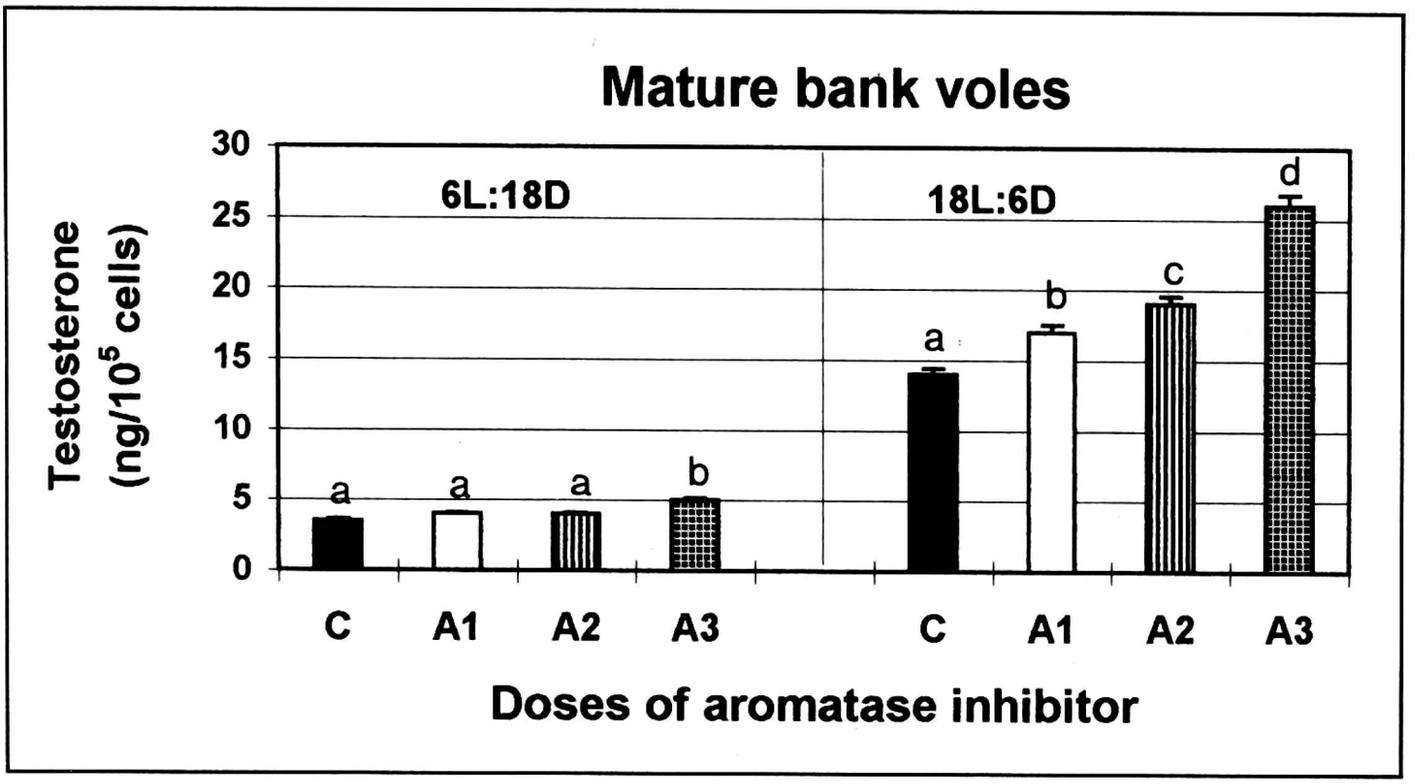



*Fig. 6.* The effect of increasing concentrations of aromatase inhibitor CGS 16949 A (A1 = 0.1 μg/ml, A2 = 0.5 μg/ml, A3 = 1.0 μg/ml) on basal testosterone secretion by Leydig cells of mature bank voles from a short and a long photoperiod. All values are mean ± SE. Means without common superscripts (a, b, c) are significantly different ($p < 0.05$).

In mature animals from a short photoperiod the basal level of estradiol was about 80 pg/$10^5$ cells, while in a long one it was 1.5-fold greater (120 pg/$10^5$ cells). The administration of aromatase inhibitor to the culture of Leydig cells from a short day did not affect the estradiol secretion. However, in case of the analogous culture from a long day only influence of the highest dose was statistically important and brought about inhibition of estradiol release (*Figs 7, 8*).

b) The Leydig cell culture medium from all groups of animals was enriched with testosterone. It resulted in a significant increase in estradiol production comparing to the basal one. Estradiol secretion by immature and mature Leydig cells from a short photoperiod was about 14 pg/$10^5$ cells and 150 pg/$10^5$ cells, while in analogous culture from a long photoperiod the estradiol level was increased to the values of 20 pg/$10^5$ and 250 pg/$10^5$ cells, respectively. Leydig cells from bank voles kept in a short day (independently of the age of donor animals), were insensitive to the administration of aromatase inhibitor, while in a long day it caused a marked decrease in estradiol secretion (especially distinct in mature males). The obtained results were of statistical importance (*Figs 7, 8*).

c) The administration of both LH and testosterone to the Leydig cell culture medium resulted in an increase in the estradiol secretion to the very high level. The maximal response was observed in the culture of Leydig cells from mature animals kept in a long photoperiod (840 pg/$10^5$ cells). In analogous culture

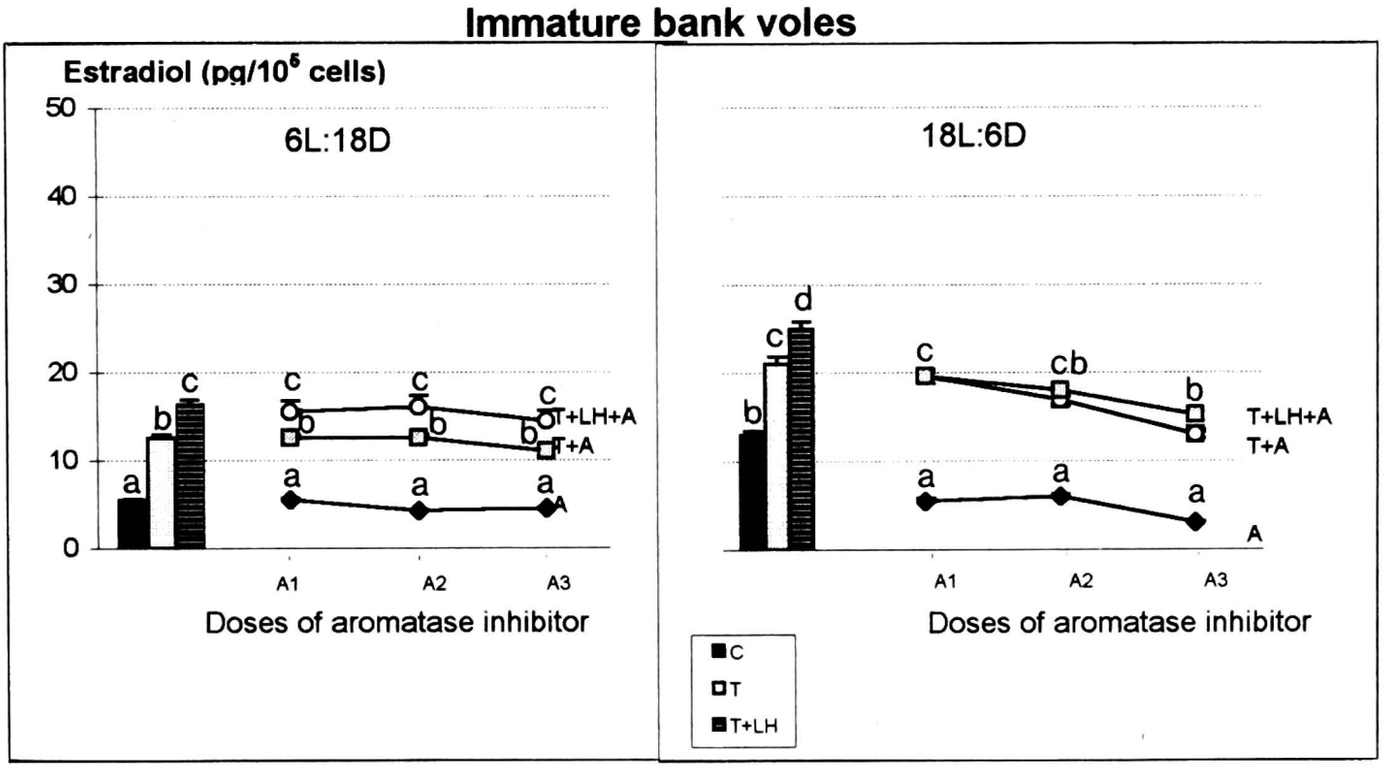



*Fig. 7.* The effect of aromatase inhibitor CGS 16949A (A1, A2, A3) on basal, testosterone--supplemented, estradiol secretion by Leydig cells of immature bank voles from both regimes of light. All values are mean ± SE. Means without common superscripts (a, b, c) are significantly different ($p < 0.05$).

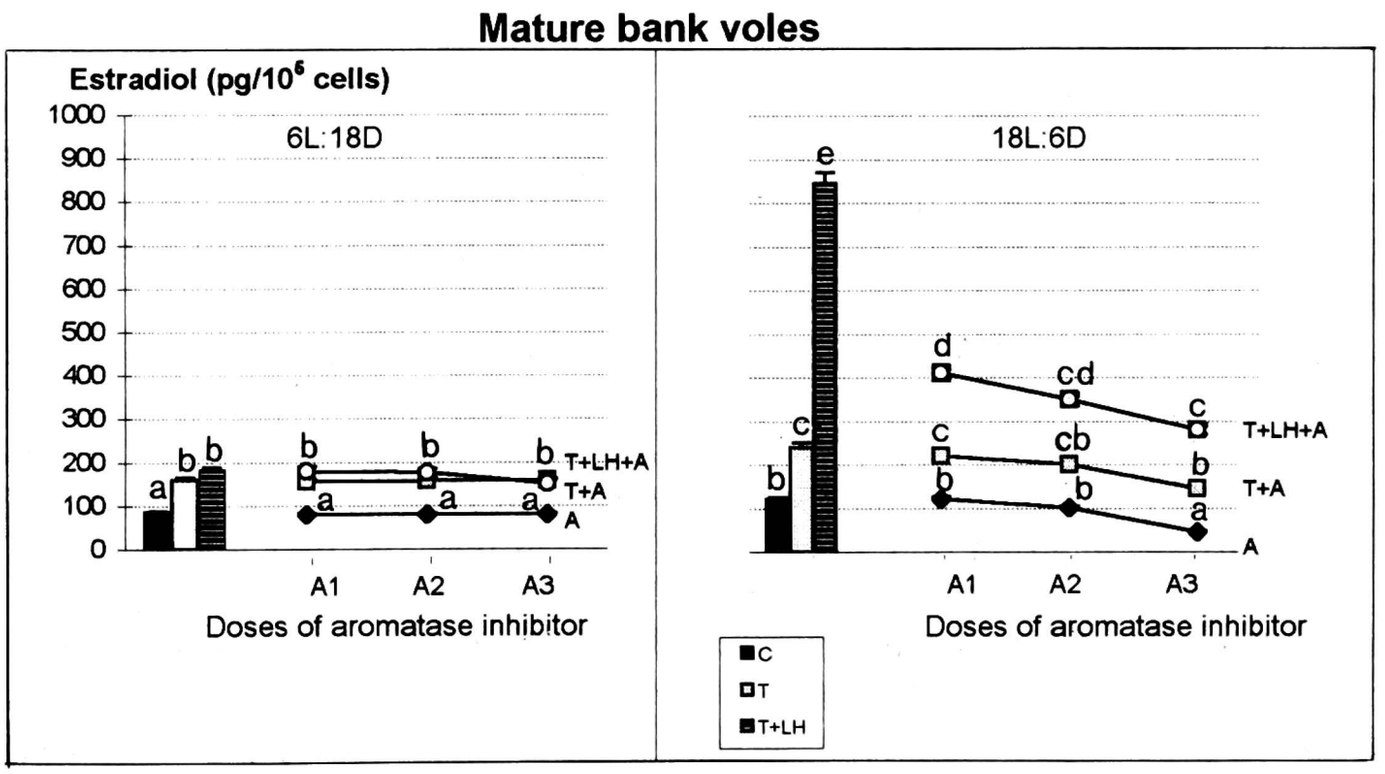



*Fig. 8.* The effect of aromatase inhibitor CGS 16949A (A1, A2, A3) on basal, testosterone--supplemented, LH- and testosterone-supplemented estradiol secretion by Leydig cells of mature bank voles from both regimes of light. All values are mean ± SE. Means without common superscripts (a, b, c) are significantly different ($p < 0.05$).

from a short day the level of estradiol secretion was much lower (180 pg/$10^5$ cells). In immature animals, despite the stimulation of LH and testosterone, the observed increase in estradiol release was slight, from 16 pg/$10^5$ cells to 23 pg/$10^5$ cells. Leydig cells from bank voles kept in a short day (independently of the age of donor animals), were insensitive to the administration of aromatase inhibitor, while in a long day it caused a dose dependent decrease in estradiol secretion especially distinct in mature males (the obtained results were of statistical importance) (*Figs 7, 8*).

## DISCUSSION

Aromatase, a cytochrome P-450 microsomal enzyme which enables the conversion of testosterone to estradiol-17 β has been immunolocalized in testes of many species. However, the precise site of aromatization in many cases is still controversial. Aromatase expression occurs in Leydig cells of human (14), rodents (15), pig (16), ram (17) and stallion (18) but it is well known, that in various animals Leydig cells are the main, but not the only source of estrogens. In mouse (19, 20), rooster (21) and brown bear (22), except Leydig cells, germ cells have been reported to show aromatase activity. Recently localization and activity of aromatase has also been detected in rat germ cells (23). In testes of adult rat and pig, principal source of aromatase are Leydig cells, although Sertoli cells have also been implicated in testicular estrogen production (localization of this proces seems to be correlated with age) (8, 9, 24, 25). In the present study we use nonsteroidal aromatase inhibitor CGS 16949A, which has been found to have a high potency and selectivity towards the inhibition of aromatase enzyme (26) to measure the ability of aromatase to convert testosterone to estradiol. This study revealed that in the testes of bank voles, Leydig cell are the main source of aromatase. However, in the culture of Leydig cells from immature bank voles aromatase level was very low, while in adult animals 50% increase in its amount was observed. The obtained results suggest that estrogen secretion is age-dependent, being higher in mature than in juvenile bank voles. This was also reported by Rommerts *et al.* (8) and Tsai-Morris *et al.* (9) in rats, Raeside *et al.* (27) in pigs and by Kmicikiewicz *et al.* (28) in mice. This data could support the hypothesis that during maturation of the testes Leydig cells come through several biochemical and morphological changes. At the end of the fetal development there is a very low level of aromatase activity and estrogen receptors. As the testis matures, Leydig cells begin to acquire the features of the adult tissue: their steroidogenic activity, the number of estrogen receptors together with the aromatase activity increase.

In seasonally breeding species steroidogenic and enzymatic activity of the testes may be also influenced by such environmental factors like

photoperiod Tähkä *et al.* (29). This study revealed that aromatase activity in bank voles was photoperiod-dependent. However, the differences in aromatase activity were seen only in case of mature tissue. In immature bank voles despite the regime of light, aromatase activity was at a very low level. In animals the length of the photoperiod had a profound effect on the aromatase activity and estradiol biosynthesis, being lower (10-fold) in the Leydig cell culture from a short day, than in the culture from a long one. Moreover, Leydig cells from a long day appeared to use greater amounts of exogenous testosterone, a potential substrate for estradiol biosynthesis than the analogous Leydig cells from a short one.

Bank voles seem to be very sensitive to a short photoperiod, which was found to induce profound changes in the morphology and activity of their testicular tissue. According to Clarke (30), in a long day, testes are large and show all stages of spermatogenesis. The steroidogenic activity of Leydig cells is very high. In a short day, testes are smaller, they have the spermatogenic epithelium reduced to spermatogonia and Sertoli cells with or without spermatocytes. Leydig cells during nonbreeding season exhibit morphological evidence of cellular inactivity, including a marked reduction in size and a marked diminution in the amount of organelles, especially the smooth endoplasmic reticulum. It all results in a much decrease in Leydig cells' output of hormones (29, 31).

The inhibitory effect of a short photoperiod on the activity of testicular enzymes and reduced androgen/estrogen biosynthesis seems to be caused by increased secretion of melatonin by pineal gland which modifies the hypothalamus-pituitary axis controlling function of the testes. Melatonin inhibition of GnRH release brings about a decrease in pituitary LH, FSH and PRL secretion and subsequent decrease in LH receptor number and P450scc activity. Low level of gonadotropins induces a quiescence phase of the testes resulting in a very low activity of the spermatogenic epithelium. This is extremely important for the Leydig cells function, as the aromatase is a gonadotropin-sensitive enzyme, which activity is closely connected with gonadotropin level. This is in agreement with previous findings done in rats (9, 32, 33).

The present study also showed different sensitivity to the stimulation of LH of young and adult bank voles kept in different regimes of light. In immature animals from both regimes of light only a slight increase in estradiol secretion was observed, suggesting a very low level of estradiol receptors. During maturation of the testis as the number of estradiol receptors increases, the growing sensitivity of Leydig cells towards gonadotropins could be observed. Thus, in a long day Leydig cells appeared to be the most sensitive to the administration of LH.

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