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Evidence for no relationship of the pineal activity with the neonatal shrinkage of Leydig cells in the rat[1]

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Summary. The collective volume of Leydig cells in prenatally pinealectomized newborn rats declined as sharply as in intact newborn rats. Also, the collective volume in pups born in the dark declined in a fashion similar to that in pups born in the light. The results indicate that neither the pineal gland nor the light is responsible for the neonatal shrinkage of Leydig cells.
Key words. Leydig cell; pineal gland; neonate; fetus, rat.

The drastic neonatal decline of the collective volume of Leydig cells has been well documented[3,4]. This decline is neither due to the disappearence of transplacental maternal LH nor to the cessation of supply of chorionic gonadotrophin (CG). Maternal pituitary LH cannot cross the placenta[5]. It has been recently shown that rat placental extract contains a trace amount of CG[6]. However, the activity of CG during fetal stages seems to be too low to maintain the growth of Leydig cells, since fetal hypophysectomy stops the growth of Leydig cells[3,7,8]. Also, the cessation of supply of maternal estrogen does not seem to be responsible for the neonatal shrinkage of Leydig cells[9].

It must be considered that the pineal gland of the newborn rat may act as a factor in the shrinkage of Leydig cells, since the pineal glands of fetal rats near term as well as of newborn rats can release some melatonin in organ culture[10]. It is well established that melatonin inhibits the output of LH by influencing the hypothalamic-pituitary system[11,12], and that the synthesis of melatonin in the pineal gland and its release occur with a diurnal rhythmic high level at night and a low level during the day[13].

In general, birth in the rat occurs during the day, not at night. It is quite possible, therefore, that the light at birth activates the pineal gland in some way, and this then produces melatonin during the 1st night following the birth. On the basis of this view, it is possible that in pups born and maintained in the dark, the pineal gland is not activated for melatonin production. The present work was designed to test these possibilities by observation of changes in the collective volume of Leydig cells following prenatal pinealectomy or following spontaneous birth in the dark.

Materials and methods. Wistar rats were fed a commercial diet (Labo MR Breeder) and water. The morning on which mating was detected by the presence of sperm in the vaginal smear was regarded as day 1 of gestation. In our rat colony spontaneous delivery occurred around noon on day 22 of gestation. The next day was counted as day 1 after birth.

In the 1st series of experiments, 6 pregnant rats were subjected to a midventral laporotomy under ether anesthesia on day 20 of gestation. Then 2 male fetuses in each litter were subjected to pinealectomy. The sex was determined by viewing the fetus through the translucent uterine wall; the distance between the genital tubercle and the anus was clearly longer in the male than in the female. For fetal pinealectomy, a fine tapered glass tube connected with vinyl tubing to a water aspirator was used. The tube was inserted through the uterine wall so as to pierce the fetal skull at a point on the sagittal suture just behind the cross of the coronal-sagittal sutures, and the fetal pineal gland was removed by aspiration. Two other male fetuses in the same litter were sham-operated. Autopsy was performed on day 22 of gestation or day 1 after birth. At autopsy, these pinealectomized and sham-operated animals were matched at random with 2 intact littermates.

In the 2nd series of experiments, 3 pregnant rats were housed individually on day 20 of gestation in a dark room and were delivered of their pups in the dark. Delivery in the dark was usually delayed. The pups born by 20.00 h on day 22 of gestation were used in this study, and the pups born later were dis-

carded. Three other pregnant rats were allowed to bear their pups under a 12 h on –12 h off light cycle. Autopsy was performed 24 h after birth, and 2 male fetuses in each litter were selected at random.

The fetuses and newborn pups were sacrificed and their testes were removed. Completeness of the fetal pinealectomy was confirmed histologically by sagittal sections of the head (figs 1 and 2). The rate of successful operations was 100%. The right testis was fixed in Bouin's fluid. Testicular volume was measured on the fixed gonad, using the formula $V = 4\pi AB^2/3$ where A was half of the longest diameter and B was half of the shortest diameter. Each fixed testis was then embedded in paraplast (Sherwood Medical Industry). Sections, 7 µm thick, were prepared serially and stained with hematoxylin and eosin. The collective volume of Leydig cells was estimated by the method of Chalkley[14]. The basis of this method is the multiplication of the testicular volume by percent of Leydig cells among random hits with pointers placed in the ocular of the microscope. Every 5th section was used. Total hits in each testis were approximately 4000. Analyses of the data were made with Student's t-test. A p less than 0.05 was considered statistically significant.

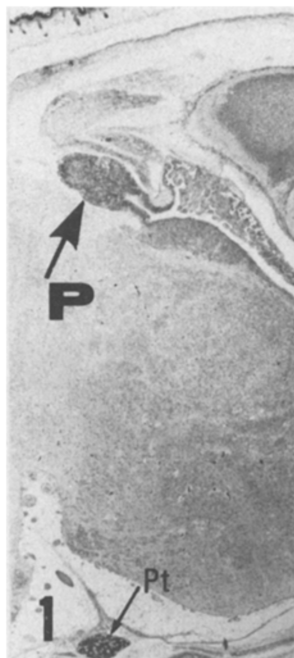

Figure 1. Sagittal section through the head of an intact newborn rat on day 1 after birth. The right side of the figure is rostral. P, pineal gland; Pt, pituitary gland. × 35.

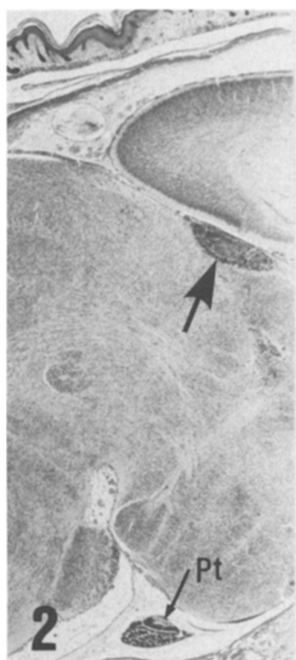

Figure 2. Sagittal section through the head of a prenatally pinealectomized newborn pup on day 1 after birth. The pineal gland is completely removed by aspiration without any serious damage of the brain. An arrow shows a nest of hemorrage induced after aspiration. × 35.

Results and discussion. In the 1st series of experiments, the collective volume of Leydig cells on day 22 of gestation was almost the same in the 3 groups (fig. 3). On day 1 after birth, the collective volume of Leydig cells in pinealectomized pups did not differ significantly from that in control pups, whether sham-operated or intact (fig. 3).

In the 2nd series of experiments, the collective volume of Leydig cells in pups born in the dark was $5.67 \pm 0.05 \times 10^{-2}$ mm^3 (mean ± SEM of 6 determinations). The collective volume of Leydig cells in pups born in the light was $5.46 \pm 0.17 \times 10^{-2}$ mm^3 (mean ± SEM of 6 determinations). These values were not significantly different from one another.

The results constitute a new line of evidence that the pineal gland has no relation to the neonatal shrinkage of Leydig cells. This view is further supported by the observation that birth in the dark had no effect on the neonatal involution of Leydig cells. The decline of the collective volume of Leydig cells just after birth can be overcome with injected gonadotrophin[3]. Therefore, this decline may be largely accounted for a decrease in the LH-releasing activity of the pituitary of the newborn pup itself. Morphologically, the pineal gland of the rat is fairly well organized, as a solid organ, at the end of gestation[15]. Electron microscopically, some cells of the gland acquire dense granules located closely to the cellular basement membrane during perinatal days[16]. These observations suggest that the pineal gland is prepared for releasing its hormone during this period. However, as the present results show, the pineal gland is not responsible for the neonatal shrinkage of Leydig cells; i.e., melatonin does not seem to be secreted in vivo from the gland on the night just following birth, though the in vitro study shows a possibility of melatonin secretion[10]. The present negative results for the role of the pineal gland in suppressing the neonatal pituitary-Leydig cell system make it necessary to seek for other explanations for the neonatal shrinkage of Leydig cells.

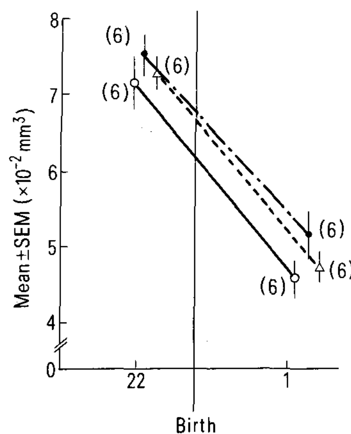

Figure 3. Graphic representation of the collective volumes of Leydig cells just before and after birth, following various treatments. Each figure in parentheses indicates number of animals. Each vertical line shows SEM; ○—○, pinealectomized; ●–·–●, sham-operated; △– – – –△, intact control.

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Defense chemicals in leaf surface wax synergistically stimulate oviposition by a phytophagous insect

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Summary. Propenylbenzenes, coumarins, and a polyacetylene, identified in the surface wax of carrot leaves, stimulate oviposition in the carrot fly. These compounds are known to be powerful inhibitors of the growth of bacteria, fungi, plants, and nonadapted herbivores. Their co-occurrence appears to be unique to the Umbelliferae, the host plant family of the carrot fly. An artificial mixture of these compounds proved to be synergistic and as stimulatory as the crude carrot leaf extract.
Key words. Leaf surface wax; defense chemicals; insect oviposition; carrot; *Daucus carota;* carrot fly; *Psila rosae.*

The number of defensive plant compounds so far identified is impressive but far from being complete[2]. There is little doubt that these secondary plant metabolites are powerful protective mechanisms against parasitic microorganisms, herbivores and competing plants[3]. By contrast, relatively few plant compounds are known which attract and stimulate herbivores during their search for suitable host plants[4]. This is especially the case for the host selection behavior of female insects that lay their eggs on or near the food-plants of their progeny. Since the larvae emerging from the eggs usually have only limited abilities to locate suitable food plants, their survival depends greatly on appropriate host plant selection by their mothers. It is surprising, therefore, that so little is known about this crucial phase in the life of phytophagous insects[5]. Unsuccessful attempts to isolate and identify the host plant compounds acting as attractants or stimulants have even led some investigators to believe that host plant specificity in oligophagous insects is primarily a consequence of the taxonomic restriction of particular feeding inhibitors[6].

Here we describe the successful identification and quantification of secondary plant components present in the crude extract of leaf surface wax, which act synergistically as a host plant recognition signal during oviposition by the carrot fly, *Psila rosae* (Diptera, Psilidae) and we offer the hypothesis that such complex mixtures of defensive plant metabolites in leaf surfaces may also be involved more generally in host selection and oviposition behavior by oligophagous insects.

The larvae of the carrot fly attack the roots of cultivated and wild Umbelliferae. Prior to oviposition females perform a typical 'run' on the leaf which is terminated as the female descends the stem of the leaf to the soil where the eggs are deposited[7]. Since females have no access to the leaf interior, their host plant selection can only be based on the shape, color and chemistry of the leaf surface[7,8]. Earlier studies showed that leaf surface compounds appeared to be the most significant stimuli for host plant selection[8]. Crude extracts of the leaf surface were prepared by dipping intact carrot *(Daucus carota)* leaves in methylene chloride twice for 30 sec each. The activity of extracts and of their fractions was tested by applying amounts equivalent to the weight extracted from fresh leaf (~ 6 g) to artificial leaves made of filter paper. The measure of activity was the difference between the number of eggs deposited at the base of the extract-treated leaves with that under the solvent treated leaves[8]. Since our extraction procedure does not disrupt the epidermal cells, the resulting crude extract probably contained little material from the leaf interior[9]. Further evidence for the presence of stimulating compounds on the leaf surface was obtained by extracting leaves with 0.1% aqueous Tween detergent, another relatively mild extraction solvent.

To isolate the active components, the crude CH_2Cl_2 extract (dry wt 330 mg) from 1.5 kg carrot leaves was subjected to 2 fractionations on silica gel columns[10]. The first separation yielded one fraction (100% Et_2O) that was active at a level equivalent to the extract of 10 g of leaves (fresh wt). This fraction was further partitioned into 2 active subfractions: 40 and 100% Et_2O. The 40% Et_2O fraction was active at 3-g leaf weight equivalent (LWE) and GC-MS analysis[11] showed the presence of *trans*-methyl-isoeugenol (**1**) and *trans*-asarone (**2**)[12]. Compound **1** has previously been isolated and identified from macerated carrot leaves[13]. Compound **2** has recently been identified in the head space above carrot leaves[14]. Each compound has been found to be attractive to carrot flies in the field[14]. The 100% Et_2O fraction proved to be active at the 1-g LWE and was separated into 20 subfractions using HPLC[15]. All fractions and subfractions were analysed by GC-MS[11].

The compounds identified in the biologically active subfractions were the substituted coumarin osthol (**3**) and the polyacetylene falcarindiol (**6**)[12]. The search for other coumarins and furano-coumarins in the subfractions and in the original silica-gel fraction (100% Et_2O) resulted in the identification of the 2 furanocoumarins bergapten (**4**) and xanthotoxin (**5**), which were active in the bioassay. Another angular furanocoumarin,