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Reproductivity and skeletal differentiation in rats housed at different temperatures

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Embriologia e morfogenesi. — *Reproductivity and skeletal differentiation in rats housed at different temperatures.* Nota di LUCIANA RINALDI e ROSANGELA CINQUETTI, presentata (*) dal Socio S. RANZI.

RIASSUNTO. — L'esposizione continuata a 28 °C, temperatura di soli 5 °C superiore a quella abituale, sembra provocare nel ratto un certo aumento dell'ovulazione (maggior numero di corpi lutei) al quale segue però una notevole e significativa perdita di embrioni pre- e post-impianto.

Un corrispondente abbassamento della temperatura ambiente sembra anch'esso stimolare l'ovulazione. L'incremento della mortalità prenatale non differisce significativamente da quella dei controlli. L'effetto nocivo dell'adattamento al freddo diventa invece significativamente evidente dopo la nascita: oltre ad una mortalità nella prima settimana più che doppia rispetto ai controlli, un elevato numero di piccoli è affetto da gravi malformazioni.

Anche l'evoluzione scheletrica pre- e post-natale risente dell'ambiente termico variato. L'ambiente « caldo » provoca ritardi nel differenziamento osseo e altera marcatamente la normale progressione nell'ossificazione della colonna vertebrale. A 18 °C invece l'ossificazione procede più rapidamente in fase fetale avanzata e nei primi giorni di vita; a partire da 4–5 giorni inizia però un rallentamento, non omogeneo, per cui a 12 giorni l'evoluzione scheletrica appare nel complesso arretrata rispetto ai controlli, e con squilibri tra le parti. Anomalie scheletriche tipiche del ceppo si manifestano con frequenza nettamente superiore.

Adverse effects of stress during pregnancy have been the subject of much research. It has been observed that stress impairs embryo-fetal morphological development [1, 2], but also interferes with biochemical and functional differentiation, thus causing alterations that become evident only in adulthood.

Ward and Weisz have found that the male offspring from female rats stressed throughout the second half of pregnancy exhibits a feminine sexual behaviour. This has been ascribed to stress-mediated modifications in the ratio of adrenal to gonadal androgens during critical stages of sexual differentiation; patterns of testosterone secretion by testes during fetal life would be, therefore, desynchronized compared with sexual differentiation of the fetal brain [3, 4, 5, 6].

Prenatally stressed female rats exhibit irregularities of estrous cycle [7] and decreased fertility and fecundity [8]. By cross-fostering experiments, Herrenkohl [8] has showed that this is to be possibly ascribed to hormonal alterations of the fetal developmental environment rather than to not proper parental care from prepartally stressed mothers.

Disturbances in hormonal balance analogous to those induced by stress, that is sudden, strong and briefly lasting changes of some environmental factors (light, temperature, living space ...), also occur in the course of adaptation to

(*) Nella seduta dell'8 maggio 1982.

environmental conditions different from the usual ones. Petrovic and Janic–Sibalic [9] have observed decreased activity of the catecholamine synthesizing and degrading enzymes as well as lowered corticosterone plasma levels in rats maintained at 34 °C for 21 days.

There is a lack of information on the influence of metabolic disturbances which occur in the course of adaptation on reproductivity and development. Increased pre- and postnatal mortality and decreased rate of growth or development have been observed in mice raised in rooms maintained at 5 and 28 °C [10]. Alterations in progesterone metabolism associated with increased fetal death-rate and lower body weight and number of pups born have been found by Bedrak *et. al.* [11] in " heat-acclimatized " rats, that is exposed to about 34 °C beginning from 5 weeks before mating. In these studies, temperatures markedly different from the usual ones were generally adopted; pre- and postnatal mortality and body weight were chiefly used as parameters to determine the effects of the environmental temperatures on reproduction and development.

Continuous but moderate changes of the environmental temperature, however, can often occur in consequence of human interferences (i.e., inside and near industrial areas). The present investigation was undertaken to determine if adaptation to thermal conditions moderately different from the usual ones also influences rat reproductivity and development; as a parameter to detect possible morphogenetic disorders, skeletal examination was adopted.

Methods

In one experiment, 120 random-bred Long-Evans rats (90 virgin females and 30 males weighing about 250 and 300 g, respectively) about 90 days old were randomly allotted to three groups housed in three different rooms kept at the following temperatures: 23 ± 1 °C (control room), 28 ± 1 °C (" hot " room) and 18 ± 1 °C (" cold " room). Both females and males were placed, as usual, in group cages housing three animals each, maintained on freely accessible diet of rat chow and water and under a natural summer light-dark cycle.

After two weeks one male was put in every female cage for 3 days; the pregnant dams were housed individually and then sacrificed by an overdose of ether on days 17 and 18 after male removal: fetuses were delivered by cesarean section and resorptions were recorded; ovaries were excised for counting corpora lutea.

Fetuses were immediately fixed in 95% ethyl alcohol and observed under a stereomicroscope to detect gross external malformations and then they were eviscerated, cleared and stained with Alizarin red S method for skeletal examination [12], in order to determine fetal age [13] as well as to detect eventual developmental defects.

The results obtained from the experiment described above led us also to investigate the effect of " cold " exposure on neonatal survival and development. In this second experiment 50 female rats were used; 24 were housed in the

control room and 26 in the " cold " one, beginning 8 weeks before mating; other rearing conditions were all similar to those adopted in the first experiment. For both control and " cold " groups the pregnant rats were housed individually and observed daily to determine the day of delivery; the numbers of viable and of dead pups were recorded daily beginning from the day of birth.

For skeletal examination all pups from two litters (one from control and one from " cold " group) were weighed and sacrificed by an overdose of ether on days 0 (day of birth), 1, 2, 4, 5, 7, 9 and 12.

RESULTS AND DISCUSSION

In both experiments no statistically significant difference was observed as to the rate of pregnant rats from control and tested groups.

In the first experiment 157 fetuses (53 controls, 68 " hot " and 36 " cold " exposed) were examined for ossification. In the second experiment 80 and 64 pups were born to control and " cold " exposed rats and observed for neonatal survival and gross malformations; 54 and 48, respectively, were examined for skeletal evolution.

Reproductivity. Counting of corpora lutea revealed a slight ovulation increase in both " hot " and " cold " groups; but whereas in the control and " cold " groups the implantations/corpora lutea ratio kept within normal limits (0.89 and 0.93, respectively), in the " hot " group the implantations/corpora lutea ratio dropped to 0.73, thus denoting that over a fourth of eggs degenerated very early in the development.

TABLE I

Reproduction data.

GROUPS	CONTROL	HOT	COLD
Fetuses alive (average No/dam)	10.6±1.34	8.5±3.77	12.0±2.00
Corpora lutea (average No/dam)	13.4±3.78	15.7±4.46	15.3±1.50
Implants (%)	89	73 (**)	93
Fetuses alive (%)	89	58 (**)	78
Total prenatal lethality (*)	10.4	46 (**)	22

(*) $= \frac{\text{No. of corpora lutea—No. of live fetuses}}{\text{No. of corpora lutea}} \cdot 100.$

(**) : $P < 0.01$.

The changed environmental conditions, however, interfere in postimplantation development of both tested groups, which showed a rate of intrauterine death at more or less late stages of about 20%; on the contrary in the control group the live fetuses/implantations ratio remained as high as 1.

These data seem to indicate that the continuous exposure of rats to altered rearing-temperatures is accompanied by increase in the ovulation rate on the one hand, but by a striking preimplantation loss (" hot " group) and postimplantation lethality (" hot " and " cold " groups) on the other hand (Table I).

But only the damage caused by the " hot " environment turned out statistically significant.

The following investigation on the " cold " exposure influence on postnatal survival supported the results of the first experiment: in fact the number of pups born to control and cold-exposed rats did not differ significantly. Nevertheless the cold environment also appeared strongly to affect survival, but its effects became evident only after birth; in addition to a mortality limited to the first week of life more than twice compared with the control one, about 12% of pups appeared to be affected by major visceral and/or skeletal malformations (Table II).

TABLE II

Postnatal viability.

GROUPS	CONTROL	COLD
Newborn alive/litter (average No.)	8.3	9.1
Viability index (*)	88.0	73.4 (**)
Grossly malformed pups alive (%)	0	12.5 (**)

$$(*) = \frac{\text{No. of pups alive on the 6th day}}{\text{No. of pups alive at birth}} \cdot 100.$$

(**) : $P < 0.05$.

Fetal and neonatal ossification. In the " hot " group fetuses a delayed appearance of the ossification centres in the skull, vertebral column and feet was seen. As previously reported for the Long–Evans rats [13], in the controls ossification of the centra began in the posterior thoracic-anterior lumbar region and then spread regularly in both a cephalic and a caudal progression; in the " hot " group fetuses, on the contrary, it first appeared in the middle thoracic centra and then advanced in a chaotic sequence, and the ossification degree and the number of vertebrae involved varied extremely both among the litters and within the litter. Anomalous progression in the appearance of the ossifi-

cation centres was also noticed in the sternum, associated with a few cases of marked sternebral dislocation.

In the " cold " group, skeletal differentiation progressed more quickly mainly throughout the last fetal period as revealed, for instance, by earlier ossification of cervical centra, which usually ossify just before birth [14].

From birth to day 4 ossification was still proceeding rapidly. Within this period the calvarian bones enlarged too fast, overlapping one another and bending their edges to the inside; most vertebral processes and all the phalangeal diaphysis became manifest, differentiation being more precocious than in the controls.

From day 4 skeletal evolution proceeded more slowly particularly in some districts, causing imbalance among the different regions.

In the 4–5 days control pups the skull bones appeared to have been carried upward and outward [15]; this is probably due to the volumetric expansion of the growing encephalon [16]. Then, from day 9 the skull bones began to establish sutures which were especially evident in the lateral regions of the skull and between splanchno-and neurocranium.

TABLE III

Feet: skeletal differentiation on the 12th day.

GROUPS	CONTROL	COLD
No. of carpal and tarsal bones...	16	12 (**)
No. of ossified epiphysis	14	8 (**)

(**) : $P < 0.05$.

On day 12 the " cold " pups still presented delayed establishment of the connections both among the neurocranium bones and between the neuro- and splanchnocranium. At this age, furthermore, the evolution of the lower jaw appeared more backward than in the controls, whereas the upper one did not, thus indicating a marked dyschrony in the masticatory apparatus development.

In contrast with a more precocious moulding of stylo—and zeugopodium and ossification of the second phalanges, occurring within the first days of life, the 12 days " cold " pups showed retarded ossification in carpal and tarsal bones, and metacarpal, metatarsal and phalangeal epiphysis; in fact, the carpal and tarsal bones and the epiphysis ossified at this stage were significantly fewer than in the control pups (Table III).

At this stage the evolution of the lumbo–sacro–caudal region also appeared to be delayed, dorsal fusion of the neural arches being observed only from sacral 1 to caudal 3 instead of from lumbar 3 to caudal 3; furthermore the arti-

cular surface of the ilia were only differentiated for sacral 1. On the contrary and unlike in the control pups, the neural arches of the atlas appeared to have fused dorsally.

Skeletal anomalies. In a previous research [15] skeletal peculiarities for the Long–Evans strain were described, such as rib 13 reduced and posterior toracic centra anomalously shaped, showing a deep ventral groove caused by missing ossification.

In the cold environment a statistically significant increase in the incidence of these variants occurred: 34% of pups displayed rib 13 reduced and 79% had imperfect posterior thoracic centra as compared to 17% and 22%, respectively, of the controls (Table IV).

TABLE IV

Skeletal anomalies

GROUPS	CONTROL	COLD
Pups with lower thoracic centra anomalously-shaped (%)	22.2	78.7 (*)
Pups with rib 13 reduced (%)...	16.6	34.0 (**)

(*) : $P < 0.001$.
(**) : $P < 0.05$.

The skeletal differentiation of this region would seem to occur in a particularly unsteady microenvironment, and therefore more subjected to influences of not proper developmental conditions.

CONCLUDING REMARKS

The results of these experiments indicate that adaptation to temperatures slightly higher or lower than the standard one affects reproductive efficiency as well as fetal and neonatal development in the rat.

The statistically significant reduction of the percentage of the implantations (Table I) in the rats housed in the " hot " room suggests that exposure to 28 °C disturbs the reproductive processes which take place before or immediately after implantation. Baumgartner and Chrisman [17, 18] have observed an increase in number of atypical and degenerate eggs in mice exposed to hyperthermic stress (i.e. to 35 °C for $15\frac{1}{2}$ h); according to these investigators this would account for the decreased reproductive efficiency in animals during periods of high

ambient temperature. The preimplantation loss as well as the increased fetal death rate and the alterations of morphogenetic processes showed by skeletal examination in the " hot " group are also to be possibly associated with the maternal metabolic disturbances occurring in the course of adaptation to a moderate heat [9, 11] which modify the developmental hormonal milieu [11].

That the reproductive efficiency is also affected by continuous exposure to a temperature lower by only 5 °C than the usual one is clearly indicated by our results. But the adverse effects of the hypothermic environment on development chiefly become manifest only after birth. In fact, although the prenatal death rate was of about 20 %, it did not turn out significantly higher compared with the controls. In the newborn pups, on the contrary, viability appeared to be markedly and significantly reduced by a high neonatal mortality as well as a high incidence of gross malformations and minor developmental defects.

On the basis of the above reported data we can presume that in the course of adaptation to a cool environment, metabolic alterations occur which could account for the observed adverse effects on reproduction and development.

Moreover it would seem that a moderate decrease of ambient temperature influences rat reproduction by mechanisms rather different than those stimulated by an analogous thermal increase.

It would be interesting to get a deeper insight into this topic, since in the available literature we are aware of papers dealing only with metabolic modifications occurring during or after adaptation to markedly low temperatures (4–6 °C).

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