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# Sexual maturation in female rats: Hereditary, developmental and environmental aspects

R. W. Rivest*

*University of Geneva, Department of Medicine, Division of Endocrinology, CH-1211 Geneva 4 (Switzerland)*

*Abstract.* Two physiological components of sexual maturation, vaginal opening and first estrus, apparently evolve similarly in Wistar and Sprague-Dawley rats. However, a bimodal distribution in the frequency of the days of vaginal opening is observed within a given strain, which is less related to heredity than to the timing and type of experiment. In addition, when the modulators of sexual maturation are reviewed, it can be observed that sensitivity to external stimuli can vary even within a strain. For a defined set of breeding conditions, one group of rats can be more susceptible to changes in the lighting regimen and not be affected by controlled stressors, while another group responds more to stress and less to light. The reason for susceptibility to one rather than another environmental factor under similar breeding conditions is not understood. In that context, it is difficult to evaluate the role of heredity when we cannot understand the full impact of the environment, not to mention maternal influence in fetal and early life. Using two lines of psychogenetically selected rats, it was possible to show that they had differences in sexual maturation, which strongly suggested a genetic predisposition. Nevertheless, the question arises as to whether the genetic locus directly affects organs implicated in sexual maturation or whether it acts on some unknown factor which only secondarily modifies sexual maturation. In summary, there is more need to understand the role of the environment, including that of the mother early in fetal and neonatal life. It is suggested that the mechanisms underlying organ growth are set for a given species, while developmental and environmental factors fix the timing of vaginal opening and first ovulation. In the rat, there appear to be two times which are preferred for vaginal opening, given the laboratory conditions that have been used in the last 20 or so years: an early period, at 31–35 days, and a late period, at 36–40 days. An explanation for this dichotomy would be that a combination of parameters (not necessarily always the same) is needed for vaginal opening. These parameters oscillate during sexual maturation with different frequencies, which can achieve resonance to lead to vaginal opening and ovulation only during given periods.
*Key words.* Development; heredity; lighting environment; melatonin; puberty; rat; Roman Low Avoidance; Roman High Avoidance; stress.

## *I. Position of the problem concerning heredity and reproduction*

The wide use of the albino rat necessitated the installation of many colonies throughout the world. In each of these colonies, selection of breeders and differences in breeding facilities led to the development of specific hereditary traits, which can be selected to give specific substrains[82]. To evaluate the hereditary component in the differences observed between rats, it is necessary to assess the amount of variation which results from ontogenic development. In the first summary of this kind concerning vaginal opening, it was observed that huge variations occurred not between strains, but within the same strain used in different laboratories: mean days for vaginal opening in different colonies of Long-Evans rats were 38, 43, 53 and 77 days; similar variations were also described for Wistar rats, with colony means at 42, 47 and 49 days[56].

Given the importance of Wistar (WI) and Sprague-Dawley (SD) rats, we centered our study on these rats, evaluating the timing of sexual maturation through assessment of vaginal opening and first estrus. Different environmental conditions were used in studies which

lasted 6 years, to evaluate within one colony and between different colonies the impact of the environment on sexual maturation. The comparison was extended to include data obtained with two lines from the same Wistar strain, psychogenetically selected on the basis of their avoidance behavior, and bred in parallel since the beginning of their selection. These rats are of the Roman Low Avoidance (RLA) and Roman High Avoidance (RHA) lines.

### *II. History of Wistar and Sprague-Dawley rats*

The rat was introduced into the laboratory at the end of the XIXth century (for a historical review, see Lindsey[47]). Later, the albino rat (*Mus norvegicus albinus*, *Rattus norvegicus*) was selected for its docility and different strains were developed, among which the Wistar rat (WI), selected by Henry Herbert Donaldson (1857–1938) at the Wistar Institute in Philadelphia. The first rats were brought to the Institute in 1906 and an inbreeding program began in 1909[47]. The Wistar rats used today are mostly randomly bred, but there are defined inbred strains (the Lew strain, the Brown Norway strain, etc.[47]). In the course of his studies, Donaldson described variations in body composition between wild *Mus norvegicus* captured in Europe and those captured in America, and between the wild rat and the albino rat (*M. n. albinus*)[14,15]. Others, for example Hatai[33], found a number of differences between the wild and the albino rat, with albino rats having adrenals and ovaries which were half the size while pituitaries were twice as big as those in the wild Norway rat. The body weight of the albino rat at that time was 45 g at 33 days[15]; a similar weight is achieved today at 20–25 days.

Around 1925, Robert Worthington Dawley (1897–1949), a physical chemist at the University of Wisconsin, established the Sprague-Dawley (SD) strain (the name is a combination of his first wife's maiden name Sprague and his own) and founded the commercial firm Sprague-Dawley Incorporated dedicated to the advancement and sale of his rats[47]. In a communication from Sprague-Dawley Inc. to the National Institutes of Health in Bethesda on July 22, 1946, it was stated that 'this strain started originally with a hybrid hooded male rat of exceptional size, and vigor, which genetically was half-white. It was mated to a white female and, subsequently, to its white female's offspring for seven successive generations. The origin of the male is unknown. The original white female was of the Douredoure strain which probably was from Wistar. After its death, its white offspring were inbred in a number of different lines from which the best ten were combined. Selection was made to retain or acquire characteristics of high lactation, rapid growth, vigor, food temperament, and high resistance to arsenic trioxide' (cited in Poiley[69]). SD rats were preferred also in cancer research because they were very susceptible to hydrocarbon-induced mammary cancer, in contrast to Long-Evans or Wistar strains[35]. From the original colony, different colonies were developed around the world, virtually all of them random-bred, with the obvious consequence that within a strain, substrains have emerged. This leads to differences that are often misinterpreted. For example, basal ACTH levels in SD rats originating from a colony in France (IFFA-Credo, L'Arbresle) are twice as high as those coming from SD rats used at the Salk Institute (La Jolla, CA), even when conditions of sacrifice are tightly controlled (C. D. Walker, personal communication).

### *III. Female reproduction*

Female reproduction is physiologically characterized by an opening of the vagina at around 30–50 days of age, which is followed by initiation of estrous rhythmicity, with its behavioral components. In order for these events to occur, the ovaries have to be able to secrete sufficient amounts of 17$\beta$-estradiol to activate opening of the vagina[21,37] and stimulate, through a positive feedback, the hypophysis[64]. Ovarian follicles must have developed to the point of releasing the first ova. At a higher level, the hypophysis has to be ready in response to the final rise in 17$\beta$-estradiol to secrete huge surges of luteinizing hormone (LH) and follicle stimulating hormone (FSH) which will trigger first ovulation.

One of the signs of maturation is the advent of a specific pattern of LH in the blood, characterized by sharp peaks, called pulses, appearing about once every 30–60 min, and by 5-fold higher and longer lasting minisurges. LH pulses are sufficient to stimulate 17$\beta$-estradiol production[98], which in turn is responsible for LH minisurges[99]. These minisurges help to stimulate final ovarian development. Pulses can be detected in 26-day-old WI rats (fig. 1) and in 27-day-old SD rats[97]. Minisurges are present as early as on day 26 in the afternoon sampling periods in WI rats (fig. 1). In SD rats, there is a diurnal pattern in LH secretion at around 30 days of age, with higher amplitude LH pulses and minisurges present specifically in the afternoon[3,97]. An increase in plasma prolactin is also detected at around 30 days of age in the afternoon, and both these LH and PRL diurnal rhythms are ovarian independent[40,64].

Complex interactions between the ovary, the brain and the pituitary thereafter establish estrous rhythmicity[38,78]. But there appears to be hormonal estrous-like rhythmicity active prior to vaginal opening. In the two weeks preceding vaginal opening, LH (and to a lesser extent FSH) secretion can be higher for one or two days on different occasions, in such a way as to mimic following estrous cycles; these rises tend to be rhythmic, as if to herald future proestrous surges (fig. 2).

#### *1. Vaginal opening in Wistar and Sprague-Dawlay rats*

Onset of puberty has been postulated to be genetically controlled. Substrains of rats have been bred on the basis

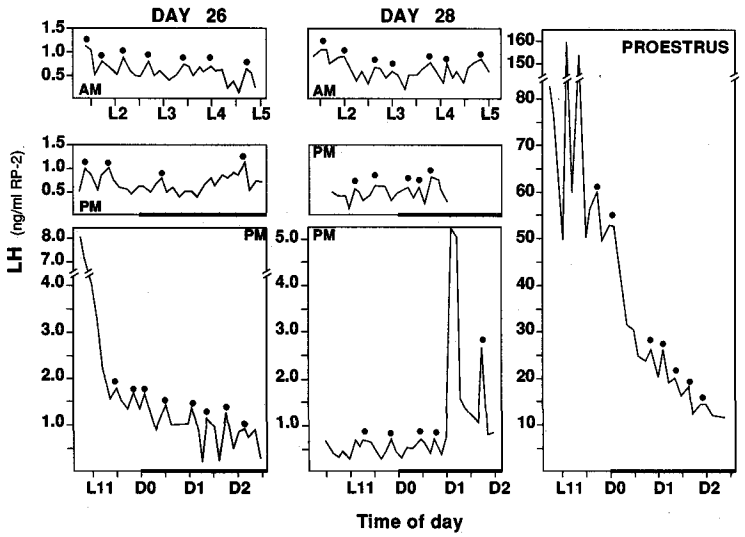


Figure 1. Description of LH pulsatile secretion early in the prepubertal period, with animals monitored either in the morning (AM) or in the afternoon (PM). The units of the abscissa represent the numbers of hours elapsed during the light period (L) or during the dark period (D, under a dim red light, less than 0.5 lux). The animals were housed in LD 12:12 (12 h of light), with lights on at 07.00 h for experiments in the morning (AM) and at 01.00 h for experiments covering the afternoon to beginning of dark period. This latter regimen had been gradually shifted before fecundation (1 h/d). The cannulation procedure was a modification of that of Urbanski and collaborators[96], with the return catheter placed in the internal facial vein[76]. The animals were operated on at 23 days of age. LH levels are expressed in terms of the NIADDK RP-2 standard, and 25 or 50 µl were assayed in duplicate. Pulses are identified with dots and analyzed with the program PULSAR, with the parameters used previously defined[80]. Minisurges are LH peaks which go higher than 2 ng/ml. For comparison, LH levels attained during the proestrous surge are presented on the right.

of the time of vaginal opening[87]. But onset is also largely influenced by exteroceptive factors. In our experiments there were more differences within a strain (WI) over a number of years than between strains (WI vs SD), thus confirming what has already been described[56].

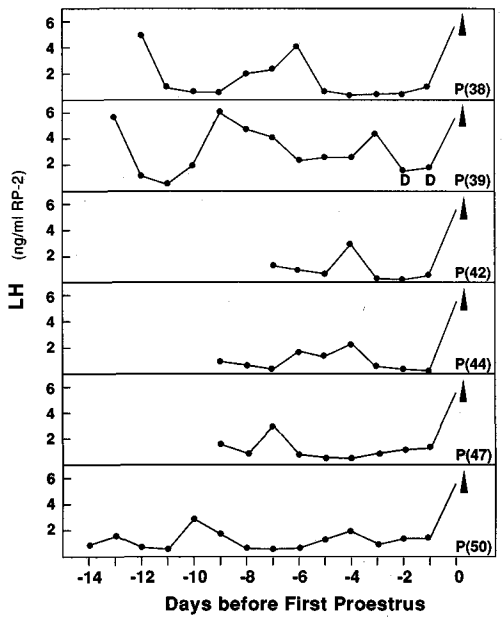


Figure 2. Description of daily plasma LH fluctuations in consecutive blood samplings taken at L10 (10 h after light onset), prior to first proestrous (P). The day of first proestrous is given in parentheses following the P, and D prior to proestrous signifies that the vagina had opened but that the animal was in diestrus. Refer to caption in figure 1 for details. Significant elevations of LH are present prior to vaginal opening, heralding future proestrous surges (see text). On the day of proestrus (P), LH levels are very high and this is represented by the arrow (see figure 1 for an example of a proestrous surge).

In the WI and SD strains, vaginal opening occurs at the same time. In figure 3, a summary of the frequency of vaginal opening established in our laboratory is given. It covers 4 experiments performed with SD rats in one year ($n = 128$), and 9 experiments with WI rats performed over 6 years ($n = 199$). There is no difference between the medians, respectively on day 37 and 39. In WI rats, our observations revealed that individual differences occurred even when the rats were bred in the same laboratory with the same food and the same lighting regimen: means (and standard deviations) of days of vaginal opening varied between $33.4 \pm 3.2$ and $41.6 \pm 3.7$, this difference being significant ($p < 0.01$ with an analysis of variance). Obviously, other parameters such as humidity and food must have played a role to provoke such differences (food composition varies according to the harvest period and region of origin of cereals, so that food can be very different despite the fact that it comes from the same company with the same catalog number). Similar conclusions can be reached for estrous rhythmicity. In all animals, we followed 3 estrous cycles. There were no differences in the estrous pattern between SD and WI rats taken as a whole, while there were individual differences among WI in different experiments, or between lines which have been genetically selected (see the section on RHA and RLA rats). We have given only the data concerning those latter rats (fig. 8).

In a WI rat population, a bimodal distribution of vaginal opening frequency was observed, with a peak at 34 days

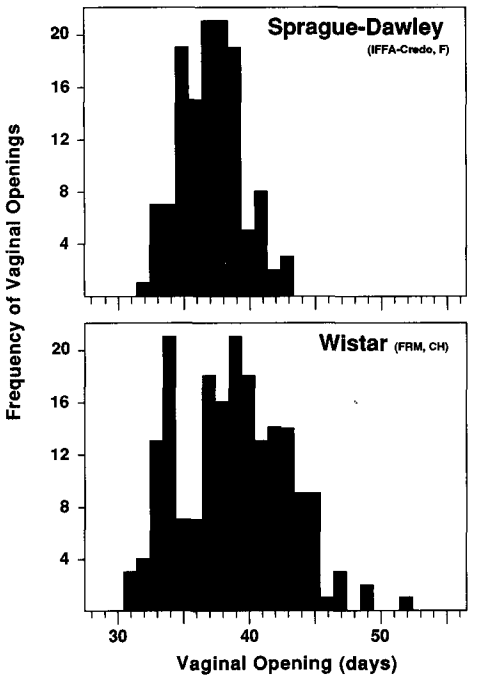


Figure 3. Frequency of vaginal openings in Sprague-Dawley (SD/OFA) rats from the colony of IFFA-Credo (France), and in Wistar (WI) rats from our colony (FRM, Switzerland). The data for the SD rats represent four experiments performed in June 1986, and the data from the WI rats, 9 experiments from November 1981 until June 1986. The housing conditions were those described in table 2.

and one at 39 days of life. For each contemporaneous litter, there was a trend for the median vaginal opening to fall on one of these two modes. Similar bimodal distributions have been observed by others, who tentatively correlated them with seasonal variations [72]. In our experiments, there was no correlation with season that was consistent over the 6 years of our study. The problem with studies on seasonal variation is that they are usually limited to two years (when they are not done in only one year!), which does not permit distinguishing between a fortuitous relation and a true seasonal rhythm. And indeed, when Ramaley did an experiment in constant light, controlling at least for possible changes in environmental illumination, the same seasonal trend was found for pubertal onset [71]. Again, other unknown factors appear to influence vaginal opening.

There could be another explanation for a bimodal distribution. Given the fact that LH secretion is oscillating at least in the two weeks prior to vaginal opening (see the previous section), and that FSH also oscillates, albeit not necessarily at the same frequency (results not presented), there could be similar oscillations in other hormones implicated in the timing of sexual maturation (no other has yet been described). One could then postulate that these different hormones enter into resonance at given periods which would correspond to the modes of distribution of vaginal opening within a population [45].

*2. Hormonal factors influencing vaginal opening*

During development, a number of variables will modify the organization of the different maturing axes. Interactions between neuroendocrine and endocrine axes are necessary for vaginal opening and estrous rhythmicity, while external conditions can set the final timing of sexual maturation. One hormonal axis is necessary for complete maturation of the pituitary-ovarian axis; that of growth hormone. Three others, those of ACTH, prolactin and melatonin, which are very sensitive to external stimuli, play a modulatory role on the LHRH-pituitary-ovarian axis.

*2.1. Growth hormone.* In the human, it was once postulated that a critical weight had to be achieved before puberty could occur [28] and, later on, this hypothesis was reformulated to emphasize the role of a threshold mass of body fat as the prerequisite for menarche [29]. In the rat, a weight of 60 g was defined as a set point under which no hormonal stimulation of ovulation could occur [107]. These hypotheses have been criticized both in the human [95] and in the rat. In this latter species, it could be shown that underfed male rats had matured spermatozoa earlier than control rats [31]. It seems more probable that a lack of sufficient nutrients affects a number of systems, and that not only one is responsible for immaturity in food-deprived rats.

Part of this maturation includes the transformation of the pattern of secretion of growth hormone, which is already secreted episodically at 22 days of age [17]. Growth hormone has been implicated in the transformation of the prepubertal form of LH into the adult form [108], an event which occurs normally when rats reach about 60 g [9], which explains this apparent causal relationship. Using a larva of the tapeworm which produces a growth factor called PGF (plerocercoid growth factor), Ramaley and Phares [73] dissociated growth from the effects of growth hormone. With PGF, growth resumed normally, but growth hormone production was suppressed. In these rats, vaginal opening and first ovulation were delayed. Replacement therapy with growth hormone restored vaginal opening to normal and partially normalized the time of first ovulation, confirming that the onset of puberty is not tightly coupled to body weight gain or growth per se. This is even further corroborated by the fact that WI and SD rats have a similar sexual maturation despite different growth rates [90].

Just like that of most of the hormones in the neuroendocrine axes, the production of growth hormone is influenced by external factors. It has a circadian organization comparable to that of the hormones to be subsequently described, with ultradian rhythms, and these rhythms can persist in conditions without time cues, even though they are normally entrained to the lighting regimen [91]. However, in contrast to that of the hormones described hereafter, GH secretion is inhibited by a variety of stressors in the rat [93], which would suggest that GH does not

mediate stress effects on sexual maturation as the others hormones appear to do.

*2.2. Prolactin.* Prolactin has also been implicated in different steps of the reproductive axis. It has an action both at the hypothalamic-pituitary level and on the ovaries. At physiological doses, prolactin at the time of puberty facilitates the development of LH receptors in the ovary [34]; it also works to maintain the LH receptor population which is required for progesterone secretion [32]. At higher doses – as obtained with pituitary transplants under the kidney capsule, prolactin implants in the median eminence, or dopamine blockade – it suppresses both the basal LH release and the pre-ovulatory type LH peaks, without affecting FSH; it increases progesterone responsiveness to gonadotropins and, finally, indirectly elevates circulating estradiol, which results in advanced vaginal opening [1, 4, 104]. Conversely, chronic suppression of prolactin delays puberty [2]. Some of these effects are due to an interaction with the adrenals, which also affect the time of puberty onset. Administration of prolactin restores the day of vaginal opening in adrenalectomized rats [30], and its action on progesterone appears to be exerted by corticosterone [1], the secretion of which it can enhance [48].

Some questions nevertheless remain unanswered. For example, administration of progesterone is known to delay vaginal opening [30] while, as we have seen, its sensitivity to gonadotropins is increased by concentrations of prolactin which accelerate vaginal opening. This suggests that the dose and the timing of progesterone presence are crucial for determining an advance or a delay, thus excluding a simple role for prolactin action on progesterone. Furthermore, it is not yet clear whether some of the effects attributed to prolactin are due to an action on dopamine in the hypothalamus. Dopamine is a neurotransmitter which inhibits prolactin but also can act on LHRH and the gonadotropins [43].

Finally, prolactin is responsive to external factors. It increases following many types of manipulations (stress) and has a circadian pattern of pulses related to the lighting regimen [85]. We have observed that unhandled pubertal control rats can have more than a two-fold increase of prolactin levels at the time of sacrifice due to a very fast (less than 45 s) stimulation of prolactin release, when compared to saline-injected rats, as has also been seen by other authors [54].

The extent of the role of these environmental variables still has to be evaluated, especially in situations where strain differences in prolactin production are observed, such as between Fisher and SD rats [19]. In addition, one has to consider the fact that in chronic stress, such as that obtained with 8-h daily immobilization for 10 days, there is a fall in prolactin (and LH and GH) in the blood [13]. The limit between an acute stressor and a chronic stressor is naturally difficult to define, and we do not know whether the sum of different daily stresses is equivalent to chronic stress. In that case, stress-susceptible rats could then be considered as living under chronic stress, with prolactin responses being inhibited in comparison to rats which would not be stressed by every external stimulation.

*2.3. ACTH and corticosterone.* Adrenalectomy performed prior to the prepubertal period (i.e. prior to 28 days) will delay vaginal opening [24], an effect that may be exerted at an ovarian site [66]. This action is counteracted by administration of corticosterone [70], which in itself does not affect puberty in intact rats [30]. ACTH administration, on the other hand, which stimulates different steroids including corticosterone and progesterone, will delay vaginal opening and first estrus, and it does not appear to act through prolactin [54]. With the synthesis of CRF (corticotropin releasing factor), it was also possible to show interactions between CRF and the GnRH-LH axis at levels higher than the pituitary, with injections of CRF blocking ovulation [81].

Thus, a direct link exists between the adrenal and the reproductive axis, and it is especially in the context of stressful conditions which elevate ACTH-corticosterone that this link interests us. In the first report on the subject, it was suggested that anesthesia or an operative procedure could accelerate vaginal opening, probably through activation of ACTH. In a subsequent study, rough handling was found to have no effect, but exposure to cold accelerated vaginal opening [55, 56]. Experiments regarding stress will be discussed later.

*2.4. Melatonin.* Melatonin, an indoleamine secreted rhythmically by the pineal gland, with the highest values occurring during the night [49], has been known to be related to reproduction almost since its discovery [109]. It was difficult to understand the role of melatonin, and of the pineal gland, until it became evident that 1) the pineal interferes with seasonal rhythms [89] and 2) it responds to different characteristics of environmental light [50]. In the laboratory rat, the main effect of melatonin is perceived at the time of puberty. Daily injections of melatonin given 9–12 h after light onset delay sexual maturation both in the male and in the female [44, 77].

In the female rat, this delay is measured both in a late vaginal opening and an even later onset of the first ovulation, with a concomitant dissociation between vaginal opening and first estrus [77]. Melatonin principally inhibits the GnRH stimulatory action on the pituitary [79], with a consequent alteration in the pulsatile LH secretion [76]. A direct action of melatonin on the ovaries is not excluded [27, 94], and it also lowers prolactin release [36].

Due to the sensitivity to the pineal parameters, including melatonin production and secretion, to numerous environmental factors, their variations have been difficult to ascribe to specific species or strains. Pineal volume can vary 4-fold within a stock of rats, and up to 300-fold between species [103]. When looking at melatonin concen-

trations in the plasma, it is not common to find differences between strains. In the rat, contents of melatonin in the pineal gland and concentrations in the blood are almost identical between Sprague-Dawley and Long-Evans rats, as is the increase following immobilization stress[51]. If there are differences between groups of animals, the most impressive differences are obtained in similar animals originating from different sources, such as different breeders or different environments. When strains obtained from different breeders were compared, it was found that Sprague-Dawley and Wistar rats from a given breeder can have pineals with similar volumes, but stocks from different breeders can differ significantly[102]. The 13-lined ground squirrel *Spermophilus tridecemlineatus* bred in the laboratory has a greater sensitivity to the inhibitory action of light on melatonin, and has a completely different diurnal rhythm from the wild animal. While the wild squirrel has a production of melatonin which covers the period of darkness, the laboratory squirrel only has a sharp peak of melatonin situated in the middle of the night[74].

To summarize the role of the different endocrine axes reviewed above, strain differences very probably exist, but they are overshadowed by environmental factors which are important in setting the timing of the events which occur during sexual maturation. This is consistent with the philosophy that a species has to develop according to the environment in order to ensure survival.

## 3. *Environmental factors influencing vaginal opening*

*3.1. Light.* One important environmental factor affecting vaginal opening is the lighting environment. Constant illumination of bright intensity advances sexual maturation: 50% or rats exposed to constant light (770 lux) from 21 days of age will have vaginal opening occurring on day 45, as against day 51 for rats housed in LD 12:12 and day 61 for rats housed in constant darkness[26].

In less drastic conditions, it is possible to see differences in puberty apparently related to seasonal changes[72], although the nature of the causal effect is not evident. In different experiments, changing the lighting regimen was not sufficient to change either vaginal opening or first estrus[11, 78]. More intriguing is the fact that 'seasonal changes' in puberty occur even under constant illumination[71], and when light, temperature and humidity are controlled for[67]. There is probably a conjunction of different environmental factors which amplify one effect. One factor which is often neglected is variation in food composition, which occurs from one batch of pellets to another, according to the natural variations in composition of wheat and other components used for food fabrication. One research group checked for estrogen contamination in food, and did not observe a seasonal variation in vaginal opening[24].

It also appears that males are more sensitive to changes in lighting regimen. Long nights (LD 8:16) delay, and short nights (LD 16:8) accelerate different parameters of sexual maturation, while females from the same litters are apparently not affected under these conditions[78]. Since light intensity is also important for synchronizing rhythms[75], experiments were performed to control both for the type of lighting regimen and light intensity. In such cases, the effect of the lighting regimen can be amplified even in females, so that differences become significant (table 1). But even this response does not seem simple, as interactions between light intensity, type of lighting regimen and spectral quality of light have been shown to delay or to advance puberty[67].

*3.3. Handling, housing, mother and litter size.* Handling, maternal influence, housing and litter size all affect maturation of the rat. Handled pups tend to have a lower basal concentration of plasma corticosterone and a smaller response of the adrenals to minor stressors (presentation of a novel stimulus); this response is similar in non-handled pups which are born from and raised with handled mothers[46]. Apparently rough daily handling from day 22 (dropping the animal one meter into a hand), had no effect on vaginal opening[56]. This experiment is not conclusive until similar 'stressful' experiments are performed earlier in the life of the pup, since the pituitary-ovarian system has reached functional maturity as early as 21 days (it can be activated by exogenous gonadotropins[12]).

The role of the mother is naturally complex, since, as has just been stated, she can influence in the foetus subsequent responsiveness to stressors in the pup. Although we do not understand why, the hierarchical position of the mother influences the number of offspring and their growth: in populations of mixed sex, the dominant females produce the most young, and the good survival of

Table 1. Day of vaginal opening and first estrus[a] after exposure to different lighting regimens with different intensities

| Lighting regimen | Vaginal opening | First estrus |
|---|---|---|
| *Lighting regimens established after birth* (on day 15 of age[b]) | | |
| A. Bright light (L = 100 lux or more) alternating with darkness (D) | | |
| LD 16:8[c] (n = 17) | 43.5 ± 5.1 | 45.4 ± 5.5 |
| | *(41–46)* | *(43–48)* |
| LD 8:16 (n = 18) | 44.3 ± 5.2* | 48.6 ± 4.5* |
| | *(42–47)* | *(46–51)* |
| B. Dim light (l = 2 lux or less) alternating with darkness | | |
| ID 16:8 (n = 21) | 37.9 ± 5.6* | 40.8 ± 8.2* |
| | *(35–40)* | *(37–45)* |
| ID 8:16 (n = 25) | 41.6 ± 6.6 | 44.0 ± 7.3 |
| | *(39–44)* | *(41–47)* |

[a] First estrus was the day the first cornified cells were observed. [b] Prior to these new lighting regimens, rats were in LD 12:12. [c] LC 16:8 signifies 16 h of light alternating with 8 h of darkness. Mean ± standard deviation; values in italics represent the calculated 95% range (SPSS program[88]). *: Differences between groups with asterisks, for vaginal opening and first estrus, were significant at the $p < 0.01$ level, using an analysis of variance with a least significant difference a posteriori test (SPSS). The experiment was done with Wistar rats (FRM, Geneva) in July.

their young contrasts with the poor survival and few progeny produced by subordinate females. In addition, direct information passes from the mother to the pup through the release of LHRH in the milk. According to Ojeda and his collaborators [64], maternal LHRH is secreted more or less continuously into the milk, and it acts as a brake on the development of the pup pituitary-ovarian axis. Without this brake, vaginal opening would occur earlier. It follows that early weaning should accelerate vaginal opening. However, in our experiments, when we compared two ages of weaning, namely weaning at 21 and at 24 days, there was no significant delay in vaginal opening in later weaners (table 2). In addition to a role for the mother, it has been proposed that the presence of an adult male could accelerate puberty, as it does in the mouse. But this does not seem to be the case for WI and SD female rats, and neither the presence of a stud nor bedding coming from adult males interferes with vaginal opening [20, 87] (the Holtzman rat could be an exception [101]). In order to understand the role of the mother better, cross-fostering experiments, which have been used when validating the lines, should again be used to control for maternal influence, but even such experiments are not sufficient to appreciate fully the maternal impact on fetal life.

Housing and litter size have more pronounced effects on sexual maturation. Under specific conditions, there is an acceleration of the time of vaginal opening when animals are housed in plastic instead of metal cages [25], or when they are bred singly after weaning as compared to animals bred in groups [63, 87]. In the first report on litter size, rats bred in litters of 2–3, 4–5 and 10–11 had vaginae opening respectively on days 47, 53 and 78 (means) [18]. It is not simple to figure out why the type of cage should influence vaginal opening. It could be an effect of differences in temperature within the cages (2.5 ± 0.5 °C higher in plastic cages in the experiment described above), despite the fact that cold (2–4 °C for 2 h, starting on day 22) has been shown to advance pubertal onset [56]. As far as litter size goes, the explanation is simpler for comparison between small and big litters. The female rat has twelve nipples, so that any litter close to and over twelve pups implies that competition for milk has to occur; for less competitive pups, there usually ensues a state of undernutrition. The reverse is true for small litters, where a state of overnutrition can be achieved. In one of the first experiments performed, it was noted that 2–3 pups per dam were overweight and had an earlier vaginal opening, at 30 days instead of 42–50 [39]. When the protein content in food was changed from 15% to 53% in the juvenile period (days 22–28), it did not affect vaginal opening [56].

A problem with litter size occurs when a comparison is made between one pup per dam and 4 pups per dam [87]. In the experiment by Slob, the animals had sufficient food and thus similar weights at the time of weaning (21 days), but the vaginae opened on day 34.7 ± 1.0 in animals housed alone as against day 38.3 ± 0.4 for groups of four (body weights were respectively of 44.7 ± 1.9 and 44.0 ± 0.8 g). Thus, differences are due to other factors. In small litters, adrenals grow bigger. Basal plasma corticosterone can be decreased [52] while stress-induced corticosterone secretion is greater in groups of four pups per dam than in bigger groups (8 and 14), an effect which persists in the adult [53]. These differences in adrenal responsiveness have been ascribed to the hierarchical stress created by the presence of other rats [92]; in a more complete study with mice, the adrenals were smaller and the body weight larger in isolated animals, while they respectively increased and decreased as the population per cage augmented to 2, 4, 8 and 16 [10]. Although corticosterone in itself does not seem to have a direct effect on sexual maturation, it could very well act in combination with other factors which are hierarchy-dependent, such as LH and FSH [8], to facilitate early sexual maturation.

Table 2. Day of vaginal opening and first estrus following different types of manipulation in Wistar rats from our colony (FRM, Geneva, CH)

| Manipulation | Vaginal opening | First estrus |
|---|---|---|
| Weaned on day 24 | | |
| Weighing q.d or q.2d | 40.9 ± 3.6 | 41.7 ± 3.7 |
| from day 15 (n = 19) | *(35–47)* | |
| Vaginal washes[a] | 33.8 ± 2.2* | 34.6 ±2.9* |
| from day 20 (n = 18) | *(32–34)* | |
| Weaned on day 21 | | |
| *No stressor* | | |
| Weighing q.5d | 38.6 ± 3.8 | 41.3 ± 3.0 |
| (n = 23) | *(31–43)* | *(37–46)* |
| *Minor stressor* | | |
| Saline injections | 39.2 ± 3.6 | 40.6 ± 4.3 |
| from day 15 on (n = 48) | *(34–45)* | *(36–48)* |
| Saline injections | 38.8 ± 4.0 | 40.4 ± 3.1 |
| from day 25 on (n = 8) | *(35–41)* | *(37–43)* |
| *Major stressor* | | |
| Jugular cannulation | 39.2 ± 3.7 | 41.7 ± 4.1 |
| on days 26–32 (n = 32) | *(36–42)* | *(38–49)* |

First estrus was the day the first cornified cells were observed. [a] Vaginae were gently flushed with warm water, using a smooth-ended glass pipette; weights were taken every day or other day (q.d.or q.2d) or every fifth day (q.5d); in the other groups, vaginae were washed once they were opened. *: Significantly different from the other groups at $p < 0.01$ (analysis of variance). Mean ± standard deviation; the values in italics represent the observed 95% range. Rats were housed in LD 12:12 (lights on at 07.00, 50–210 lux, temperature 21–24 °C, humidity 40–65%), and were fed ad libitum (U.A.R., AQ4, 91369 Epinay, F). Breeders were removed prior to birth, and litters had 9–13 pups per dam.

*3.3. Stress.* During acute stress in the rat, even with minor stressors such as handling, there is a stimulation of the release not only of ACTH, but also of prolactin, and a decline in the release of growth hormone and LH [41]. These modifications can be explained in part by an interplay of hypothalamic releasing factors, endorphins and catecholamines [58]. These conditions result in an inhibition of ovulation, and the neuroendocrine interactions are important because ovulation can occur even when LH is inhibited, for example after restraint stress [13, 59]. Given the knowledge that the environment and stress can influence sexual maturation, an attempt was made to

understand the causes for the bimodal distribution we had observed in Wistar rats. Wistar rats ordered from other colonies, which had to travel to Geneva early in life, had vaginal openings which were distributed around the first mode (fig. 4; in the two graphs, the median falls on day 32). In the two experiments described here, travel occurred at different ages, 2–3 days or 10–12 days. Presumably, travelling at 2–3 days old should not have impinged upon the adrenocortical development of the rats, since at that age, the hypothalamic-pituitary-adrenal axis is under tonic inhibition with an impaired response to stress[105]. Notwithstanding this presumption, an attempt was made to look at the effect of stress in our controlled conditions. In these experiments, even major stresses such as those following jugular cannulation as early as day 21–22 of life did not modify either the day of vaginal opening or that of first estrus (table 2). In fact, contrary to expectation, it is not our subjective appraisal of the quality of a stress which most affects LH secretion: when the effect of breaking a leg (!) was compared with that of 30 min immobilization, only the latter inhibited pulsatile LH secretion in ovariectomized rats[6]. It is difficult to differentiate between hereditary and developmental effects, especially those which result from the interaction with the mother as early as the fetal stage. Adrenocortical reactions of rats and mice in response to a new environment or other types of stressors depend on previous housing conditions[7,68]. In our hands, a major stress (cannulation procedure), which was without effect on Wistar rats from our colony, significantly delayed vaginal opening when applied to Wistar rats from a colony in France (table 3).

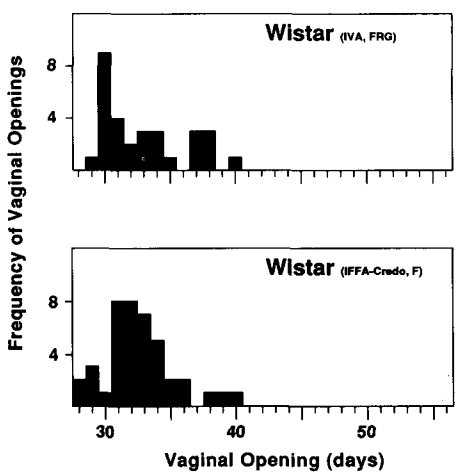


Figure 4. Frequency of vaginal openings in WI rats from two colonies, one in Germany (Ivanovas, Kisslegg im Allgäu), and one in France (IFFA-Credo, L'Arbresle). Rats from Germany were shipped by train to Geneva in a normal freight wagon in a day, at 2 days of age, and those from France, in a temperature-controlled truck in two days, at 9 days of age. Once in Geneva, the housing conditions were those described in table 2. The data from German rats represent one experiment (June 1982), and that from France, two experiments (October 1985 and February 1986).

Table 3. Day of vaginal opening and first estrus following different types of manipulation in Wistar rats from another colony (IFFA-Credo, F)

| Manipulation | Vaginal opening | First estrus |
|---|---|---|
| *No stressor* | | |
| Weighing q.5d (n = 5) | 30 ± 3 | 31 ± 3 |
| | (*28–43*) | (*29–43*) |
| *Minor stressor* | | |
| Saline injections | 33 ± 2 | 34 ± 2 |
| from day 15 on (n = 21) | (*29–36*) | (*31–36*) |
| Saline injections | 34 ± 3 | 34 ± 3 |
| from day 25 on (n = 5) | (*28–40*) | (*28–40*) |
| *Major stressor* | | |
| Jugular cannulation | 37 ± 3* | 38 ± 2* |
| on days 23–24 (n = 7) | (*32–40*) | (*36–41*) |

Rats were received in Geneva on day 10 of age, after two days of travelling in a temperature-controlled truck. They were weaned on days 21–23. First estrus was the day the first cornified cells were observed. Mean and standard deviation; the values in italics represent the observed 95% range. *: Significantly different from the other groups at $p < 0.01$. Same housing conditions as in table 2.

*4. Dissociation of vaginal opening and first estrus*

At the time of vaginal opening, the first ovulation occurs, and this event is followed within a day by an estrous phase. However, these two physiological events can be dissociated. This has been shown after adrenalectomy which depressed incidence of ovulation[24], but also after melatonin treatment which delayed vaginal opening by 10 days and first ovulation by more than 15 days[77]. Changes can also occur even under normal conditions where the type of housing is changed: for example, putting rats in plastic cages advances vaginal opening but not ovulation, when compared to rats placed in metal cages[25]. Differences in light intensity, in humidity or in stress, which can be related to the adrenals or the pineal, could explain these variations. One explanation given is that there is a rise of estradiol at the time of puberty which is sufficient to provoke final maturation of vaginal epithelium, but is not of sufficient amplitude to elicit the LH and FSH surges necessary for ovulation[78]. This is confirmed by experiments where testosterone implants (silastic capsules) in juvenile females mimicking proestrous elevations will advance vaginal opening but not first ovulation, very likely through an aromatization of testosterone at the level of the vaginal epithelium[64]. In the melatonin model where melatonin is injected daily from day 15 of age at a given time of the day, 9–12 h after light onset, this split between vaginal opening and first estrus results from an impairment at a level above that of the pituitary[78,79] and apparently does not implicate prolactin or progesterone (unpublished results).

*5. Patterns of estrous rhythmicity*

Following first ovulation, estrous cycles are maintained with a period of 4–5 days, although longer cycles can be present. Different environmental factors also alter the period of the cycles. The change from 4- to a 5-day cycle depends in part on circulating estradiol, with 5-day cycle rats showing a more prolonged and more gradual rise in

serum estradiol before ovulation, with a longer period of cornification at estrus[61,62]. But a role for prolactin has also been demonstrated, where the estrous rise of prolactin secretion is responsible for maintaining a corpus luteum life span of 3 days in 5-day cyclic rats[100]. The luteotropic action of prolactin is closely correlated with the luteolytic action of LH. Blockade of the biological effects of LH on diestrous day 1 results in a 1-day lengthening of progesterone secretion by the corpus luteum[83]. In these animals, prolactin removal on the afternoon of estrus induces a 1-day shortening of progesterone secretion[84]. In fact, Van der Schoot and Uilenbroek[100] have suggested that the lengthening from 4 to 5 days is the consequence of an activation of progesterone by prolactin, and is not due to a late follicular maturation.

The type of cycle often reflects fecundity. Hence, rats with 5-day cycles are more likely to become pseudopregnant[22], probably due to the facilitating effect of progesterone. Interestingly, they also show a higher pregnancy rate[57], possibly following a longer period of estrus.

Strain differences have been noted in the estrous rhythmicity. The sensitive period for pentobarbital to block ovulation in the afternoon of the day of proestrous was different in two strains of rats (Charles River CD vs Osborne-Mendel)[23]. There may, however, be similar differences even within one strain if, for example, one separates 4-day and 5-day cyclers: the time of onset of complete cornification in Holtzman 4-day cyclers (18 rats out of 44) was at 03.40 h ± 1.2 h (proestrous at 21.00 h and estrus at 09.00 h), while it was advanced to 20.00 h ± 1.9 h (proestrous at 09.00 h and estrus at 05.00 h) in 5-day cyclers (22 rats)[57].

### *IV. Study of two Wistar rat lines; RHA and RLA rats*

Selection of a specific character is commonly carried out by genetic breeding. Such breeding has been done, for example, using vaginal opening as a criterium for genetic selection[87]. Our research included Wistar rats which had been psychogenetically selected on the basis of their conditioned avoidance of shock stimuli[5]. Roman High Avoidance (RHA) rats rapidly learn to avoid the stressful stimulus, while Roman Low Avoidance (RLA) rats develop a freezing behavior as a means to cope with the stressor. The lines we have used were renamed RHA/Verh and RLA/Verh, after they had been bred at the Institut für Verhaltenwissenschaft in Zürich since 1972[16].

In terms of reproduction, these rats have been known to have an inverse correlation between the pineal and the pituitary (C. Gentsch, personal communication). RLA/Verh rats have big pituitaries and small pineals in comparison to RHA/Verh rats which have small pituitaries and big pineals, resulting in higher plasma melatonin levels in RHA/Verh rats[86]. We confirmed these differences, which were present during puberty (day 31 of age) and later on in adulthood (3rd estrous cycle) (fig. 5). In addition, female RLA/Verh rats had a slowing down of body growth in comparison to female RHA/Verh, and when sexual organs were weighed, after a correction for these changes in body weight, it was observed that RHA/Verh rats had smaller uteri. This last difference suggested lower basal blood levels of 17β-estradiol in RHA/Verh rats.

When blood hormones were measured, RLA/Verh females were characterized by elevated levels of prolactin, concomitant with lower levels of melatonin (fig. 6). FSH, which was higher in RLA/Verh females just prior to vaginal opening, had normalized with respect to RHA/Verh females by the 3rd estrous cycle. Basal LH levels, excluding a study on pulsatile activity, were similar in both groups. Physiologically, RLA/Verh rats had an earlier vaginal opening (fig. 7; $p < 0.05$ using a Mann-Whitney non-parametric test [SPSS grogram[88]]), with a median

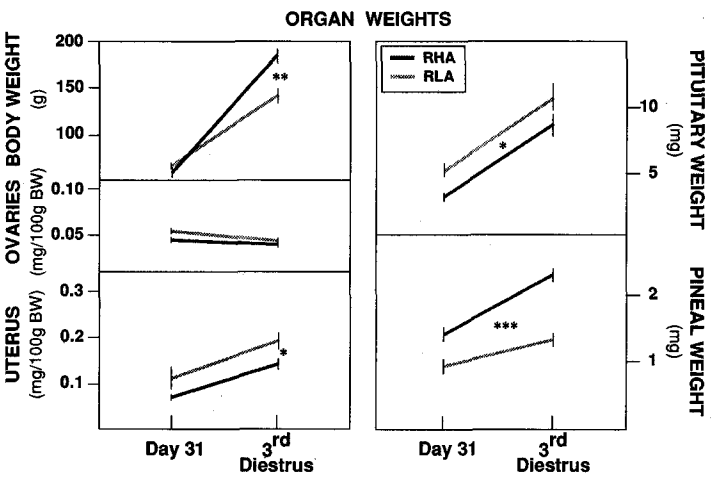


Figure 5. Organ weights in Roman High Avoidance (RHA/Verh, black line) and Roman Low Avoidance (RLA/Verh, gray line) female rats, with measures taken on day 31 of age or on the diestrus day of the 3rd estrous cycle. Animals were sacrificed between L2–L4 (2–5 h after lights on, in the morning). Because of a difference in body weight on the third diestrus, ovarian and uterine weights were expressed per 100 g b.wt. Differences are significant with a $p < 0.05$ (*) or $p < 0.01$ (**) between groups on a given day (stars under the means) or on both occasions (stars in the middle of the lines) (analysis of variance).

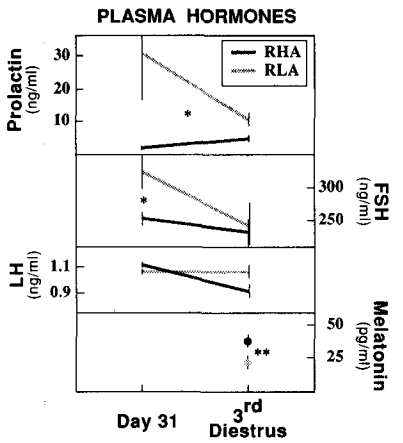


Figure 6. Plasma levels of prolactin, FSH, LH and melatonin in RHA/Verh and RLA/Verh female rats on two different occasions, at 31 days of age and on diestrus of the 3rd estrous cycle. Melatonin measurements were performed only on that latter occasion. See legend of figure 5 for details. *$p < 0.05$, **$p < 0.01$.

on day 34 (range = 31–50) against a median for RHA/Verh rats on day 46 (range = 43–56), confirming results presented by Conrad Gentsch at the European Neuroscience Association Meeting (1983), with RLA/Verh at 34.9 ± 1.8 and RHA/Verh at 41.0 ± 3.0. As for differences in uterine weight, this accelerated vaginal opening in RLA/Verh rats suggested higher basal levels of circulating 17β-estradiol.

However, RLA/Verh females rats were not simply precocious in their sexual development. Considering the estrous rhythmicity which followed vaginal opening, it was noted that 6 rats out of 15 had interrupted estrous cycles after the first ovulation, lasting more than 15 days (fig. 8). In RHA/Verh rats, all rats were cycling either immediately after vaginal opening, or at the latest after 7 days. Thus, RHA/Verh rats were more regular, an observation confirmed by their slightly greater facility for becoming pregnant (P. Driscoll, personal communication). Interestingly, the RHA/Verh rats with a delayed vaginal opening can be partially compared to our colony rats which have a delayed vaginal opening following melatonin administration, because in both cases melatonin levels are higher while prolactin levels are lower[36, 78]. There is, nevertheless, a difference in that estrous rhythmicity is impaired after melatonin treatment, which is the opposite in RHA/Verh female rats.

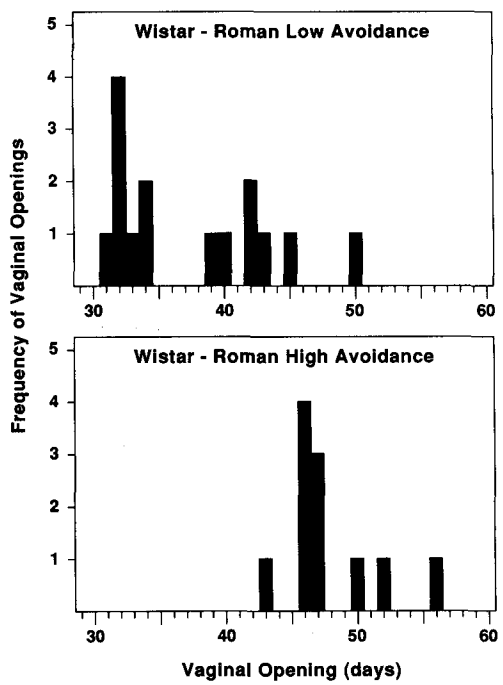


Figure 7. Frequency of vaginal openings in Roman Low Avoidance (RLA) and Roman High Avoidance (RHA) rats. The housing conditions were those described in table 2. Rats were transported from Lausanne to Geneva in 1 h on day 7 of age, and were weaned on day 23. On day 31 of age, RLA/Verh females weighed 63 ± 1 g, and RHA/Verh females, 69 ± 1 g, this difference being significant ($p < 0.01$).

## *V. Conclusions*

In the female, initiation of ovulation is a primordial step for survival of the species. It was impressive to see how easily this first step of estrous rhythmicity in the rat varies between individuals, and between groups. We sought to explain part of this variation, and necessarily included heredity as a determining factor for the timing of first ovulation.

In the human, such an approach to the hereditary component of a behavior or an endocrine function usually implies a study on homozygotic and dizygotic twins. To use an example taken from endocrinology, it can be shown that the heritability index for variability of plasma cortisol is 50%, while the remaining 50% is dependent on developmental and external factors[60]. But it is one thing to look at one endocrine axis in homozygotic twins, and another to understand a physiological event in strains of rats.

Strain differences have been studied ever since rats were first bred in the laboratory. In establishing a line, comparisons are worthwhile especially if syngeneic controls are still in the same laboratory as the line genetically selected, thus controlling for at least some of the environmental factors. However, when one studies strains obtained from different breeders, the exact impact of genetic background, as opposed to developmental and external influences, is so hard to isolate that some of these studies are almost impossible to interpret.

By consistently studying the same parameters in the same Wistar strain under the same conditions over 6 years, we have observed important differences for the two physiological parameters under study, vaginal opening and first estrus. And as we have stated, in certain experiments there was less difference between different strains of rats studied together than within the same strain studied on different occasions. This applies, for example, to Wistar rats from the same colony, with vaginal opening occurring at times as disparate as 33 days or 41 days (group means). Our conclusion is that the heritability index for

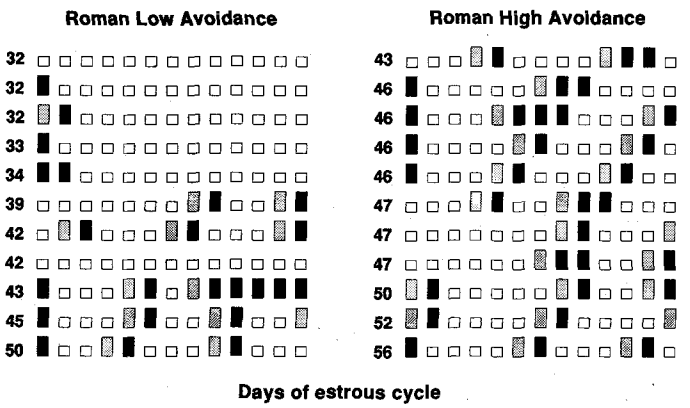


Figure 8. Day of vaginal opening (number at the left) and patterns of estrous cycles in 11 Roman Low Avoidance (RLA) and 11 Roman High Avoidance (RHA) rats. A gray rectangle represents a day with a proestrous smear, a black rectangle is a day with an estrous smear (ovulation occurs between these two days), and the white squares represent metestrous and diestrous smears. Note the lack of estrous cycles in at least 6 RLA/Verh rats, even after opening of the vagina and a first ovulation. Animals are those described in figure 7.

the timing of vaginal opening is low compared to the impact of the mother or the environment. Before assessing the hereditary component of a behavior or a variable between strains, we can only urge repeated study of one strain over two or three years in the conditions of the laboratory, before two strains are compared.

A statement such as 'since these two strains were kept under identical conditions for 2–3 weeks preceding the experiment, it is very likely that the differences observed are of genetic origin and not related to differences in housing or stress'[65] should be reconsidered. Since rats with a given genetic background can develop differently following altered breeding conditions, then for two stocks of rats originating from two colonies, it is not proven that differences in a physiological parameter involve genes. In the example cited above, physiological changes could be explained by a perception of the environment which is altered following different breeding conditions, and which persists in the controlled conditions of the study. In that sense, two genetically selected lines, bred continuously together, provide a better model for the study of genetic differences[106].

There are differences, however, between the SD and the WI rats, the most striking in our context being the difference in body growth. This difference is well documented in major breeding companies[90], and attempts have been made to correlate modification in body growth with thyroid hormones, corticosterone and prolactin, all of which can differ between the two strains[42]. As stated in the last-mentioned article, and confirming our criticisms, differences in body weight are reproducible but those in endocrine parameters are not. Prolactin levels, for example, can vary in opposite directions, and susceptibility to administered prolactin can be different between the two strains[42, 65]. Practically, these hormonal differences compensate to give similar vaginal openings.

Thus, vaginal opening in a population of rats is determined mostly by developmental and environmental factors, but for given substrain in a given environment, genetic differences can be potentiated. Moreover, there is not only one endocrine combination to sustain a given sexual maturation, but different ones, which act in combination with environmental factors.

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* Present address: Dept of Endocrinology, Laboratory M.N.S., C.P. 347, CH-1211 Genéva 4 (Switzerland).

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