A Comparison of Plasma Prolactin Levels in Young Female Long-Evans and Holtzman Rats as Measured by Nb2 Lymphoma Bioassay and Radioimmunoassay (43284)

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Abstract. **This study was conducted to determine the plasma levels of prolactin in prepubertal and young, postpubertal, proestrus rats of mammary tumor-susceptible (Sprague-Dawley) and tumor-resistant (Long-Evans) strains using a sensitive bioassay-Nb2 lymphoma cell replication. Prepubertal Long-Evans rats had significantly higher levels of prolactin than did Holtzman Sprague-Dawley rats of the same age. Likewise, Long-Evans rats secreted significantly more prolactin into the blood on the afternoon and evening of proestrus than did Holtzman rats. Finally, ovariectomized Long-Evans rats released more prolactin into the blood at 1 day, but not at 8 or 15 days, of treatment with diethylstilbestrol. Prolactin levels determined by conventional radioimmunoassay and by bioassay were similar except on the afternoon of proestrus, when, in both strains of rats, the bioassay to radioimmunoassay ratio increased significantly above 1.0 during the late evening. In addition, the ratio was significantly less than 1.0 in the early and late afternoon in the Holtzman rats, but not Long-Evans rats. These data indicate that a strain of rats that is resistant to experimentally induced mammary cancer has higher prolactin levels in the blood than does a strain that is susceptible to mammary cancer at a time when mammary gland growth is rapid. Furthermore, there are times during the proestrus prolactin surge when the bioassay yielded higher and lower values of prolactin than radioimmunoassay of the same samples, suggesting functional heterogeneity of prolactin that may impact on mammary gland or other target tissue function.**
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The importance of prolactin in the initiation and growth of experimentally induced mammary tumors in the rat has been well-documented, and comprehensive reviews of the evidence have been published by Welsch and Nagasawa (1) and Welsch (2). It is clear that prolactin enhances growth of carcinogen-induced mammary tumors. On the other hand, it has also been shown that prolactin may protect the mammary gland from the carcinogenic effects of drugs like 7,12-dimethylbenz[*a*]anthracene if prolactin is elevated naturally or experimentally prior to the administration of the carcinogen (3–6).

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Mammary tumor induction by carcinogens is also dependent on the strain of rat studied and the age at which the carcinogen is administered. Sprague-Dawley rats are quite susceptible to carcinogen-induced mammary tumors, whereas the Long-Evans rat is very resistant (7, 8). Carcinogens like 7,12-dimethylbenz[*a*] anthracene are most effective when administered to susceptible strains of rats at 10–20 days after the onset of puberty, and this is apparently due to rapid cell division in the mammary gland at this time (9).

We (7), and others (10), have shown that strains of rats that are susceptible to mammary tumor induction have different patterns of prolactin secretion than those strains that are resistant to mammary tumor formation. This is particularly true in rats at the time when carcinogen is most effective. The present study was conducted to further examine the patterns of prolactin secretion in mammary tumor-susceptible and -resistant strains of rats before and just after the onset of puberty, and to characterize the biological activity of prolactin using a mitogenic bioassay—the Nb2 lymphoma cell replication assay (11, 12).

Materials and Methods

Weanling female Holtzman Sprague-Dawley and Long-Evans rats (23 days old) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and were housed 10 per cage in an environmentally controlled room (20°C, relative humidity 40–50%, lights on 0600–2000 hr). One group of 100 rats (50 of each strain) was sacrificed by rapid decapitation at 3-hr intervals (10 rats of each strain/interval) from 0600 to 1800 hr on Day 30 of age to obtain trunk blood, which was collected in heparinized tubes. A second group of 200 (100 of each strain) was sacrificed at 2-hr intervals (10 rats of each strain/interval) from 1200 to 2400 hr on the day of proestrus (as determined by vaginal smears taken between 0800 and 1000 hr) on Days 45–60 of age, and trunk blood was also collected in heparinized tubes. A third group of 20 (10 of each strain) was bilaterally ovariectomized under ketamine (80 mg/kg)-xylazine (4 mg/kg im) anesthesia on Day 44 of age and implanted subcutaneously with a 10 mm Silastic capsule containing crystalline diethylstilbestrol (DES; 12–15 mg). These rats were briefly anesthetized with ether, and a 1.0-ml orbital sinus blood sample was obtained between 0900 and 1030 hr on Days 45, 53, and 60 of age using heparinized capillary tubes.

The plasma collected from all blood samples was stored in two separate aliquots at −20°C until assayed for prolactin by conventional double-antibody radioimmunoassay (13) and Nb2 lymphomal cell bioassay (11). All samples were assayed at two or three dilutions in duplicate, and rat prolactin NIDDK-RP-1 (11 IU/mg) was used as standard in both assays. The two assays were done within the same week and no sample was thawed more than once. In both the radioimmunoassay (RIA) and the Nb2 bioassay (BA), dilution curves for plasma were parallel to the standard prolactin curve. Care was exercised not to use more than 1 μl of plasma per milliliter of cells in the bioassay.

The effect of time was assessed statistically using one-way analysis of variance and Tukey's HSD multiple comparison test. Stains were compared at each time interval using an F test (14).

Results

Plasma levels of prolactin in 30-day-old, prepubertal Holtzman and Long-Evans rats are shown in Figure 1. Between 0600 and 1500 hr Long-Evans rats had prolactin levels that were significantly greater than those of the Holtzman rats when measured by either RIA (Fig. 1A) or Nb2 bioassay (Fig. 1B). There were no significant fluctuations of prolactin in the Long-Evans rats across the 12 hr of the experiment, whereas Holtzman rats showed a significant elevation in plasma prolactin levels at 1800 hr, compared with the other times of the day. Figure 1C shows the calculated BA to RIA ratio of prolactin across the experiment. It can be seen that the ratio varied somewhat across time between 1.0 and approximately 2.0, but there were no significant effects of time, nor were the two strains different from each other.

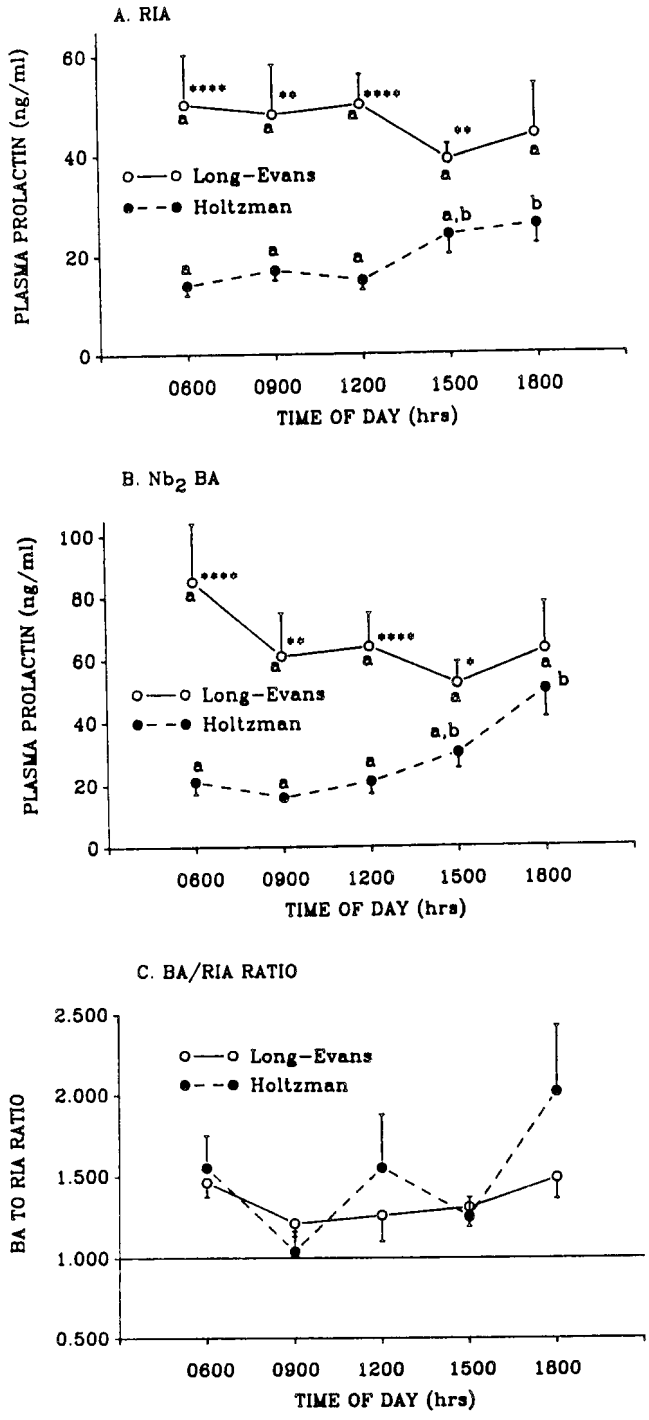

Figure 1. Plasma levels of prolactin in 30-day-old prepubertal female Long-Evans and Holtzman Sprague-Dawley rats. (A) Radioimmunoassay. (B) Nb2 rat lymphoma cell bioassay. (C) BA to RIA ratio. The solid horizontal line represents a BA to RIA ratio of 1.0. (a, b) Means compared across time within strains with different superscripts were significantly different by analysis of variance and Tukey's HSD multiple comparison test ($P < 0.05$). Means compared between strains at each time interval using an F test were different at $P < 0.025$ (**) or $P < 0.001$ (****) ($n = 10$ rats/strain/time interval).

Figure 2 shows the plasma levels of prolactin on

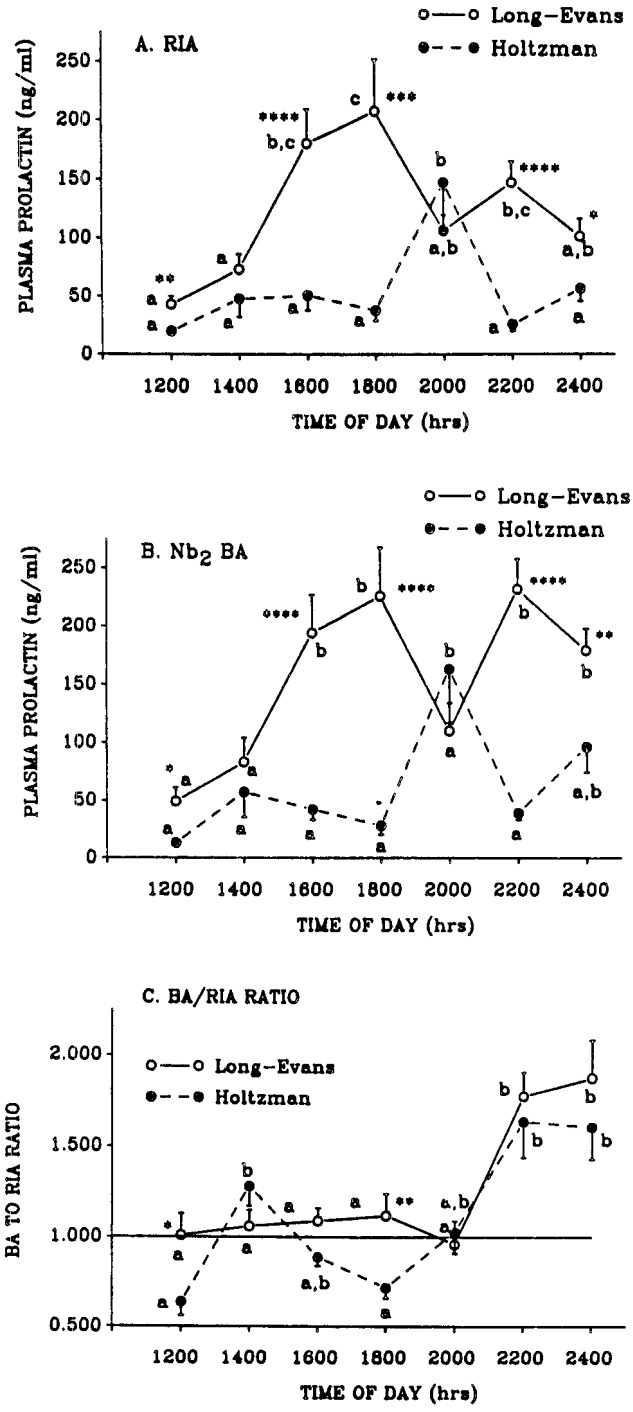

Figure 2. Plasma levels of prolactin in 55- to 65-day-old female Long-Evans and Holtzman Sprague-Dawley rats on proestrus. (A) Radioimmunoassay. (B) Nb2 rat lymphoma cell bioassay. (C) BA to RIA ratio. The solid horizontal line represents a BA to RIA ratio of 1.0. (a,b) Means compared across time within strains with different superscripts were significantly different by analysis of variance and Tukey's HSD multiple comparison test. Means compared between strains at each time interval using an F test were different at $P < 0.05$ (*), $P < 0.025$ (**), or $P < 0.001$ (****) ($n = 10$ rats/strain/time interval).

the afternoon of proestrus in 55- to 65-day-old rats of the two strains. Both strains of rats showed an afternoon surge of prolactin. However, the pattern and magnitude of the surges were quite different. Long-Evans rats had a broad surge that began between 1400 and 1600 hr, was biphasic in nature, and continued until at least 2400 hr. On the other hand, Holtzman rats had a very brief monophasic surge between 1800 and 2200 hr. These patterns were very similar when either assay was used (Fig. 2A vs 2B). The BA to RIA ratios of prolactin were constant at 1.0 in the early part of the surge in Long-Evans rats (Fig. 2C), but late in the surge (2200 and 2400 hr), the ratios significantly increased to approximately 2.0. A similar pattern was seen in the Holtzman rats, except that there was a significant increase in the ratio at 1400 hr that was not seen in the Long-Evans rats. In addition, the ratios in the Holtzman rats were less than 1.0 at two points during the course of the experiment (1200 and 1800 hr).

Plasma levels of prolactin in ovariectomized, DES-treated rats are shown in Figure 3. In Long-Evans rats, plasma prolactin levels were approximately 200 ng/ml after 24 hr of DES treatment and did not change over the course of 15 days. Holtzman rats, however, had plasma levels of prolactin that were significantly less (approximately 70 ng/ml) than those of Long-Evans rats at 1 day of DES treatment, but plasma prolactin significantly increased over the 15 days of the experiment such that by Day 8 of treatment, the levels were not different from those in Long-Evans rats. This same pattern was obtained by Nb2 BA, except that levels at 15 days of DES treatment in Long-Evans rats were significantly greater than on Day 1. The ratio of BA to RIA was not affected by DES treatment, nor was the ratio different between strains. Indeed, the ratio was constant at approximately 1.0 throughout the course of DES treatment.

Discussion

The data from this study show that rats of the Long-Evans strain, which are resistant to formation of carcinogen-induced mammary tumors, release significantly more prolactin into the blood during the prepubertal period, during the proestrus afternoon surge in the early postpubertal period, and at 1 day of treatment with estrogen, compared with rats of the Holtzman Sprague-Dawley strain which are susceptible to carcinogen-induced mammary tumor formation. Furthermore, the study shows that plasma levels of prolactin measured by lymphoma bioassay can be significantly higher or lower than levels measured in the same samples by RIA.

That prepubertal Long-Evans rats had significantly higher levels of plasma prolactin than did Holtzman Sprague-Dawley rats suggests that the former strain may have evolved a secretory profile of prolactin at a time in its life that protects the mammary tissue from the effects of carcinogenic compounds like 7,12-dimethylbenz[*a*]anthracene. We have shown previously that the circadian patterns of prolactin release were well-organized into two daily surges in the 30-day-old Long-

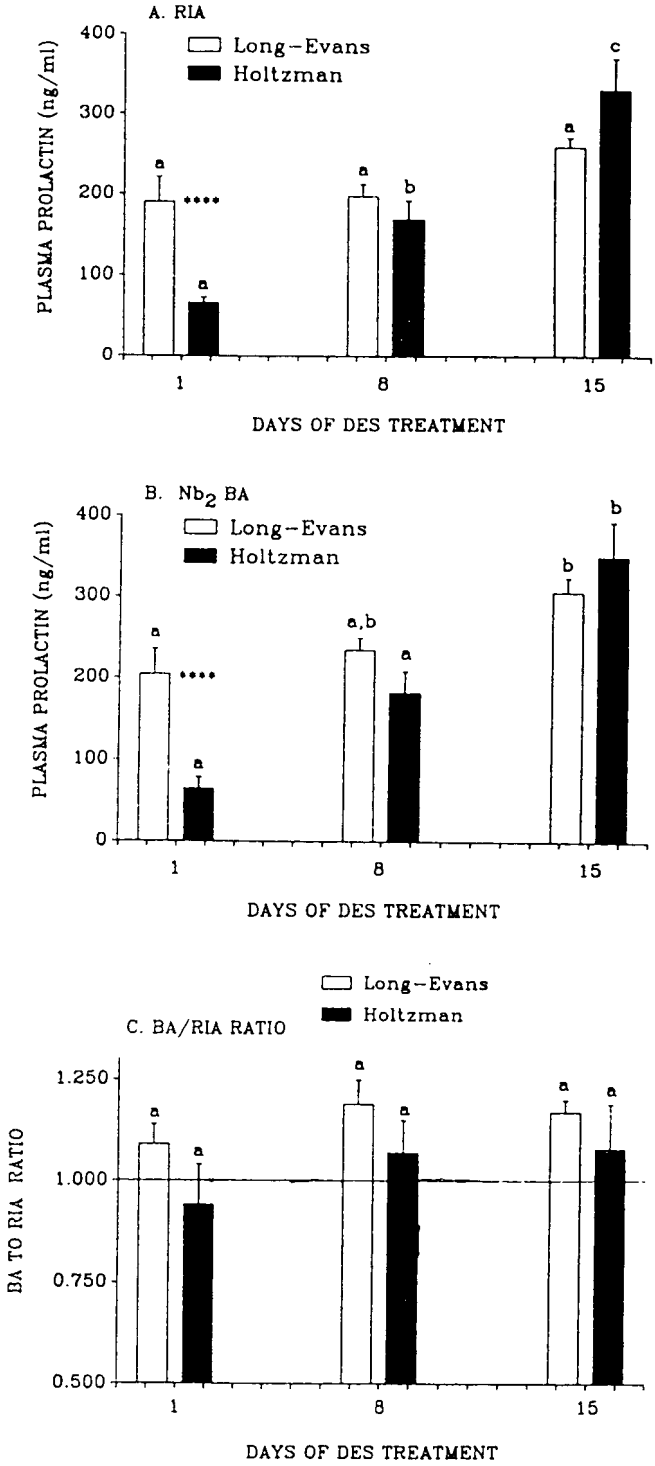

Figure 3. Plasma levels of prolactin in 45- to 60-day-old Long-Evans and Holtzman Sprague-Dawley rats ovariectomized and treated with diethylstilbestrol. (A) Radioimmunoassay. (B) Nb2 rat lymphoma cell bioassay. (C) BA to RIA ratio. The solid horizontal line represents a BA to RIA ratio of 1.0. (a,b,c) Means compared across time within each strain with different superscripts were significantly different by analysis of variance and Tukey's HSD multiple comparison test. Means compared between strains at each day of treatment using an F test were different at $P < 0.001$ (****) ($n = 10$ rats per strain).

Evans rat, but such surges were not well-organized in Sprague-Dawley rats of the same age (7). The current data did not show a circadian pattern comparable to that shown in our earlier report, but the full 24-hr day was not examined in the current study. The current and previous observations are relevant, since it is known that experimentally induced elevations of plasma prolactin reduce the incidence of mammary tumors in rats if accomplished prior to the period when carcinogen is most effective (10–20 days after the onset of puberty) (3–6). It is thought that this protection may result from prolactin-induced differentiation of mammary cells such that they do not exhibit a phase of rapid cell division at a time when carcinogen is normally most effective (Day 45–60 of age) (9). In fact, it has been shown that Long-Evans rats do not have a period of rapid mammary cell division at this time in life (8).

That Long-Evans rats released significantly more prolactin than did Holtzman rats during the afternoon and evening of proestrus in the early postpubertal period (Fig. 2) suggests that the pattern seen during the prepubertal period extends to the only other time in the reproductive cycle of the virgin rat when prolactin is released in any appreciable amount and could have a significant effect on the ultimate development and/or differentiation of the mammary gland. Perhaps the large magnitude and long duration of the proestrus surge in the Long-Evans rat compared with Holtzman rats is the result of enhanced responsiveness to circulating estradiol, which is known to induce proestrus surges of both prolactin and gonadotropins (15). In the current study, Long-Evans rats released significantly more prolactin in response to 24 hr of DES treatment than did Holtzman rats (Fig. 3), but we have no evidence concerning the effects of the natural estrogen estradiol at physiological concentrations, nor do we have evidence about estradiol-induced prolactin surges in ovariectomized rats of these two strains. Experiments to answer these questions are being planned. There is one report (16) of a comparison of plasma levels of estradiol in Long-Evans and Sprague-Dawley rats that shows young Long-Evans rats (60–65 days old) to have lower serum estradiol levels than Sprague-Dawley rats of the same age. However, the investigators did not indicate the day of the estrus cycle when the samples were obtained.

The observations that plasma levels of prolactin measured by bioassay were not always equal to those obtained by RIA suggest that the pattern of greater or lesser biological activity may be important to the ultimate response by the mammary gland. Unfortunately, the bioassay that is currently available to measure blood levels of hormone is not an assay of mammary mitogenesis or mammary differentiation, which would be most appropriate to resolve this issue. However, the Nb2 bioassay is a mitogenic assay and may provide some insight into the role of prolactin in mammary

tumorigenesis. It is interesting that the BA to RIA ratio was significantly different between the two rat strains only during the afternoon of proestrus, and then the difference was due to a low ratio (<1.0) in the Holtzman rats (Fig. 2C).

In a previous report (7), we showed that the molecular forms of prolactin released to thyrotropin-releasing hormone were different between Long-Evans and the more susceptible of the two Sprague-Dawley strains used, such that Long-Evans rats released a greater proportion of larger molecular forms than did Sprague-Dawley rats. We have recently shown that these large forms are more biologically active than the monomeric form (unpublished). These observations and the current observation that the BA to RIA ratio changed across time during the proestrus afternoon prolactin surge indicate that prolactin being released into blood is not functionally homogeneous. In addition, we also have unpublished data that show that thyrotropin-releasing hormone treatment *in vivo* increases the BA to RIA ratio in DES-treated, ovariectomized rats and that the dopamine agonist bromocryptine decreases the ratio, suggesting that release of prolactin with more or less bioactivity can be controlled by hypothalamic factors.

The current observations that the BA to RIA ratio was near 1 until very late in the proestrus afternoon surge, at which time the ratio increased to 2.0, are in disagreement with the earlier report of Blank *et al.* (17), who reported a constant ratio of 4.0 across the entire proestrous surge. The reason for the disagreement between the two studies is not clear. One possibility is that the volume of serum used in the assay by Blank and co-workers may have been sufficient to contribute serum factors which can potentiate prolactin bioactivity—an effect which we have reported previously (18). However, the report of Blank *et al.* included data that prolactin antiserum could eliminate all the Nb2 cell-stimulating activity in their serum samples. They also reported that adding purified human growth hormone to samples in which prolactin was absorbed with antiserum gave a response that was predicted (i.e., the human growth hormone response was not potentiated by factors in the serum). These observations suggest that serum factors are not responsible for the differences between their study and our current experiments. Another possibility is that the age of the animals affected the response. We have shown that diethylstilbestrol treatment of mature ovariectomized rats induces a rapid increase in the BA to RIA ratio of plasma prolactin to approximately 2.5, but this effect of DES is absent in 45- to 60-day-old animals (unpublished). The animals in the current study were 55–65 days of age when examined, but it is not clear what the ages of the rats used by Blank and colleagues were; they may have been older. It is of interest that an earlier report by Klindt *et al.* (19) showed large increases in the BA to RIA ratio on metestrus and diestrus, but not on proestrus. This later study, however, utilized samples drawn at short intervals over a 3-hr period, and it is not clear from the report when this 3-hr period occurred during the day.

Our current observations suggest that endocrinologists interested in the signaling functions of prolactin *in vivo*, whether at the mammary gland or some of the other targets for prolactin, must not only be concerned about the general level of prolactin in blood, but must also consider that there are patterns of plasma prolactin, the levels and bioactivity of which change over fairly short periods of time and do so in ways that are not the same in different strains of the same species. Parenthetically, those endocrinologists interested in the neural regulation of prolactin should also consider that all three of these aspects of prolactin may be selectively controlled by the neuroendocrine mechanisms of the hypothalamus, and that this control may not always be identical in different strains of the same species.

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