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Influence of gender and estrous cycle in the forced swim test in rats

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Abstract

The present work aimed at studying the influence of the estrous cycle in the forced swim test, an animal model of depression. For this, 44 male and female Wistar rats were divided into five groups according to the hormonal state in the first day of the study: metaestrus (N = 12), diestrus (N = 8), proestrus (N = 7), estrous (N = 6) and males (N = 11). They were housed in groups of five, with water and food *ad libitum* under a 12/12 h light/dark cycle. Females were screened daily for the estrous cycle. The animals were subjected to two swimming sessions in a glass cylinder with water up to 15 cm at 28±2º C. The data of the first five minutes of a 15-min first session were compared to those of a 5-min second session 24 h later. The results indicate that the latency to the first immobility was substantially reduced in the second session and was longer for females in diestrus and proestrus in the first session. The results also indicate that females in diestrus and proestrus exhibited less immobility than males in the first session; females in diestrus also exhibited less immobility than females in metaestrus. Females in metaestrus and diestrus, as well as males, did not present the decrease in total immobility times in the second session. The present results are analyzed in terms of differential effects of progesterone and estrogen on a learning component and an affective component. **Keywords:** Forced swim test, estrous cycle, sex differences.

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Introduction

Studies on the influences of sex differences on behavior are scarce and, in general, present contradictory and disperse data. The modulation of female behavioral states by sex hormones (in rodents, the estrous cycle) has only recently been properly approached. This is an important matter for pharmacology and human medicine, since many drugs have their effects altered by gender and hormonal condition. Besides, many psychiatric disorders are gender-dependent, such as the increased occurrence of depression and anxiety in women (Holden, 2005). Also, Klink, Robichaud and Debonnel (2002) present data on natural differences between males and females in 5-HT activity, with males exhibiting more active neurons in raphe nuclei.

Studies on the behavioral effects of the menstrual cycle in humans and estrous cycle in rodents investigate a variety of reproductive (Carter, 1993; McCarthy & Becker, 1993) and non-reproductive behaviors which influence cognitive abilities (Kimura, 1992; Dreher et al., 2006), exploratory and motor activity (Kennet et al., 1986; Curzon, Haaren & Kennett, 1990; Alonso et al., 1991; Blanchard et al., 1993; Morgan, Schulking & Pfaff, 2004), defense reaction and defensive attack (Blanchard et al., 1980; Morgan et al., 2004), as well as learned helplessness (Gouveia Jr., 2001). Few studies report on the relations between the estrous cycle and behavioral tests like the elevated plus-maze (Gouveia Jr. & Morato, 2002), learned helplessness (Jenkins et al., 2000) and elevated T-maze (Gouveia Jr. et al., 2004).

The forced swim test (FST), also known as behavioral despair (Porsolt et al., 1977, 1978), is considered an

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animal model of depression and is mainly used in the screening of antidepressant drugs (Sánchez & Meier, 1997; Petit-Demouliere, Chenu & Bourin, 2005). It is usually carried out by exposing an animal to two forced swimming sessions 24 h apart in a cylinder with water. In the first session, a rat is submitted to the inescapable forced swimming and, after an initial period of vigorous activity, the animal adopts the typical posture of immobility. This stressful situation is repeated the next day and the immobile posture occurs earlier. Measures of the onset and duration of immobility or floating are a score of "depressive-like" behaviors, which are decreased by antidepressant drugs and by other methods to treat depression (Porsolt et al., 1977, 1978). There is some evidence demonstrating that both variables are dissociable in terms of what they index; while the total duration of immobility is taken as a score of affective effects, the latency for the first episode of immobility is taken as a score of contextual/emotional learning and memory effects (West, 1990).

The FST produces an acute release of ACTH and corticosterone by the hypothalamus. In addition, FST procedures increase serotonin release in the hippocampus – an effect that is probably mediated by the activation of corticotropin-releasing hormone activity (Linhorst et al., 2002) – and raise extracellular concentrations of 5-HT in the rat striatum, while diminishing 5-HT levels in the amygdala and lateral septum. At the present moment, it is not possible to determine which of those effects are due to chronic stress and which are due to acute stress. However, the role of serotonin in mediating depression, anxiety and stress has long been acknowledged (Graeff et al., 1996). Deakin and Graeff (1991) suggested that the dorsal raphe-amygdala and dorsal raphe-frontal cortex serotoninergic pathways facilitate conditioned fear, while the DRN-periventricular pathway inhibits innate fight/flight/freeze reactions. The medial raphe nucleus innervation of the dorsal hippocampus seems to mediate resistance to chronic, unavoidable stress (Graeff et al., 1996). Nonetheless, 5-HT1A receptor binding and 5-HT1A mRNA expression were not observed in any hippocampal region after exposure to the FST (López et al., 1998). The FST also stimulates the expression and release of oxytocin and vasopressin in the hypothalamus (Wotjak et al., 1998, 2001). There are a few sex differences in the neurophysiological and neurochemical effects of the FST. Forced swimming induces a reduction in serotoninergic activity in the hippocampus and hypothalamus of female rats, but increases 5-HT activity in those same structures in male rats. 5-HT1A mRNA levels in females' hypothalamus also decrease, while 5-HT1A mRNA levels in the hippocampus increase in male rats (Drossopoulou et al., 2004).

Proestrus present less immobility than males in the first session. This report, however, used a square container for the water, which may have modified the behavior. Another example is the paper by Maryan, Chavez-Chavez and Santana (1996), using repeated testing of females (no males were tested) and reporting that, with repetition, an increase in immobility occurs during diestrus as compared to estrus.

Using another two-session model of depression, learned helplessness, Jenkins et al. (2000) compared females in estrus and diestrus, males and ovariectomized females previously treated with either progesterone or β-estradiol. In the first day, rats were subjected to inescapable tail shocks; in the second, to an escape test in the shuttle box. Results showed more learned helplessness (as measured by longer latencies to escape shocks) by females in diestrus than in estrus. In ovariectomized females, progesterone increased escape latencies while β-estradiol decreased them, indicating the influence of the estrous cycle and the importance of ovarian sex hormones.

The effects of ovarian sex hormones in ovariectomized rats points out a correlation between treatment with sex hormones and the amount of immobility in the FST (Stoffel & Craft, 2004). In another study with the same test, the administration of estrogen caused immobility to decreased immobility in addition to altering the fos-like immunoreactivity in the anterior cingulate, lateral orbital, retrosplenial, forelimb, parietal and temporal cortices, as well as tenia tecta, anterior claustrum, endopiriform nucleus, anterior and posterior dorsomedial striatum, the shell of the nucleus accumbens, bed nucleus of the stria terminalis, basolateral and medial anterodorsal amygdaloid nuclei, hippocampal CA1 and CA3, centromedial, paraventricular and rhomboid thalamic nuclei, and paraventricular, lateral, ventromedial and dorsomedial hypothalamic nuclei of ovariectomized female rats (Rachman et al., 1998).

The aim of the present study was to investigate the influences of the estrous cycle of rodents on the immobility measures of the behavioral despair test as compared to that of males.

Methods

Subjects

Wistar-derived male and female rats (± 200 g) were obtained from the animal housing of the Universidade Estadual Paulista at Botucatu. The females had their estrous cycle monitored by vaginal smears for 14 days. The vaginal smears were obtained by delicately introducing a blastus with saline solution into the vagina, smearing it on laminulae and then inspecting it in an optical microscope. The females were divided into five groups tested according to their hormonal state in the first day of testing: metaestrus (N = 12), diestrus (N = 8), proestrus (N = 7), and estrus (N = 6). Females with irregular estrous cycle were discarded. At the same time, eleven males were tested; male subjects were handled in a similar fashion as females. All animals were housed in groups of 5-6 in the same room, with free water and food under a 12/12 h light/dark cycle (lights on at 7:00 a.m.) with environmental temperature kept between 24 and 27^{O} C. All tests were carried out between 08:30 and 11:00 a.m.

Apparatus

The tests were carried out using a 40 cm high transparent glass cylinder 16 cm in diameter, filled up to 15 cm with water at 28±2º C. The sessions were recorded with a video camera (Panasonic RZ315, Brazil) and the behavioral data were later recorded with the Etholog 2.2, a software for recording behavior (Ottoni, 2000). In the present paper, we used Porsolt's definition of immobility (Porsolt et al., 1977, 1978), as the animal floating in the water and making only those movements necessary to keep the head above the surface.

Procedure

The animals were studied in two sessions that begun with the rat being gently placed into the water in the cylinder. The first session lasted 15 min while, the second lasted 5 min (Porsolt et al., 1977, 1978). Number of fecal boluses, latency for the first immobility episode and total immobility (floating) time were the parameters recorded, and comparisons were made between the first 5 minutes of the first 15 min session and the total 5 min of the second session.

Statistical analysis

One-way ANOVAs (followed, whenever appropriate, by Duncan's test to compare group means) were used to detect effects of the estrous cycle on the first 5 minutes of the first 15min session and the total 5 min of the second session. In addition, Student's *t*-test was used to compare males with females. In all cases, P < .05 was used.

Results

There were no general differences between males and females in the latency to the first immobility in the first (*t*[42] = 1.919, P =.062) and second sessions (*t*[42] = .421, P = .676). Figure 1 shows the latency to the first immobility episode in the first and second sessions by male and female rats (Panel A: Sex differences in latency for first immobility episode; Panel B: Estrous cycle effects on latency for first immobility; Panel C: Difference in latency for first immobility between training and test). There was a main effect of estrous cycle on the latency to immobility in the first session (F[4,39] = 5.226, P = .002), but not in the second one (F[4,39] = .139, P = .967). The *post hoc* test indicated that, in the first session, females in diestrus and proestrus took longer to enter immobility than males and females in metaestrus (P=.002). The *t* tests comparing the first and second sessions indicated significant decreases in the latency to the first immobility for males (*t*[20] = 2.457, P = .023) and females in diestrus (*t*[14] = 4.319, P < .001), proestrus (*t*[12] = 4.370, P < .001) and estrus (*t*[10] = 3.472, P = .006), but not metaestrus (*t*[22] = 1.310, P = .204).

Figure 2 shows the total amount of immobility in the first 5 min of the first and the second sessions by males and females (Panel A: Sex differences in total amount of

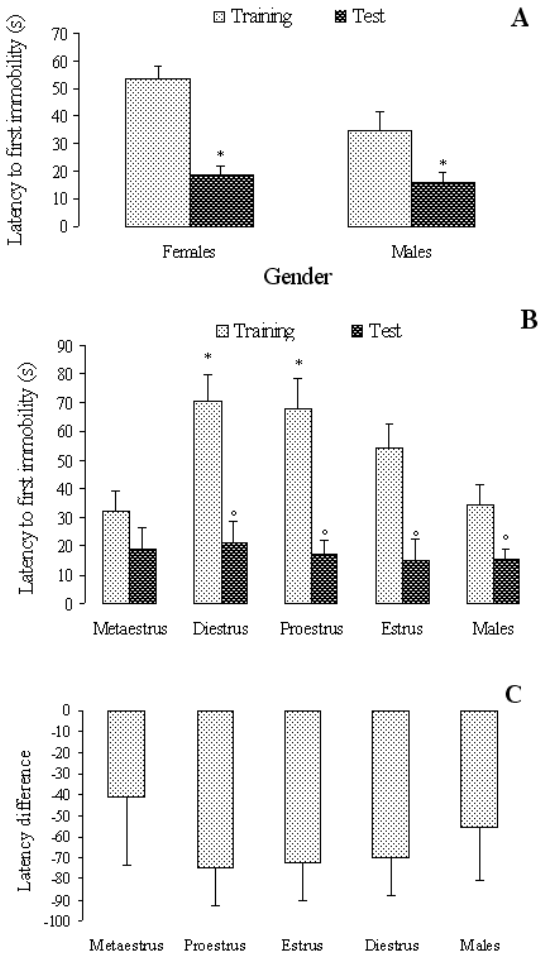

Figure 1. Latency of the first immobility period (s, means) by males and females in different phases of the estrous cycle. Panel A: Sex differences in latency for first immobility episode; Panel B: Estrous cycle effects on latency for first immobility; Panel C: Difference in latency for first immobility between training and test.*, Different from males and metaestrous (Duncan, p < .05); °, Different from training (Student *t* test, p < .05).

immobility; Panel B: Estrous cycle effects on total amount of immobility; Panel C: Difference in total immobility time between training and test). Main effects were observed in the first session (F[4,39] = 4.605, P = .004), but not in the second one (F[4,39] = 1.023, P = .408). The *post hoc* test indicated that, in the first session, females in diestrus presented less total immobility than males and females in metaestrus (P=.004). It also showed that females in proestrus and diestrus presented less total immobility time than males (P=.004). The *t* tests comparing the first and second sessions indicated significant increases in the total immobility time by females in diestrus (*t*[14] = 3.383, P =.004) and proestrus (*t*[12] = -3.343, P = .006), and no differences by males (*t*[20] = -.726, P = .477) or females in estrus (*t*[10] = -2.170, P = .055) and metaestrus (*t*[22] = -.899, P = .379).

A main effect in the number of fecal boluses was also

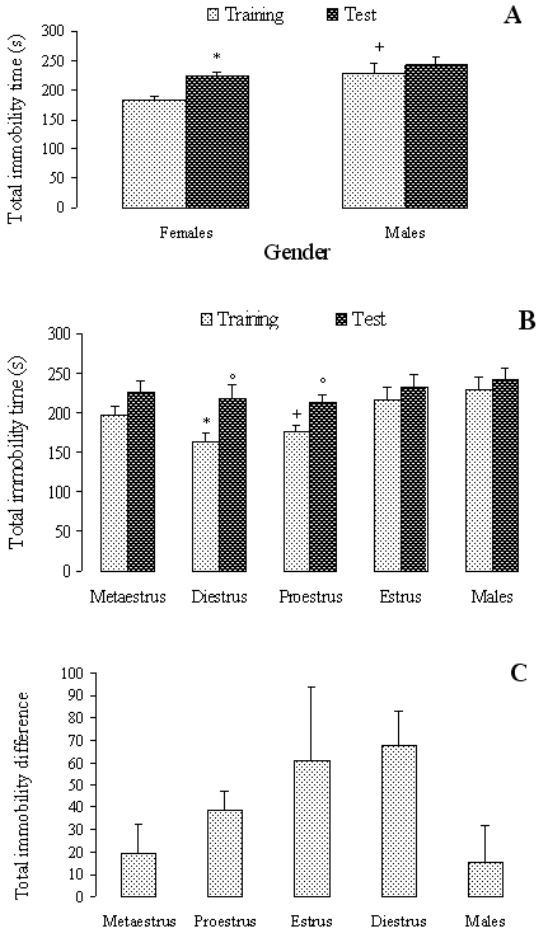

Figure 2. Total immobility time (s, mean) by males and females in different phases of the estrous cycle. Panel A: Sex differences in total ammount of immobility; Panel B: Estrous cycle effects on total ammount of immobility; Panel C: Difference in total immobility time between training and test.*, Different from males and metaestrous; $^{+}$, different from males (Duncan post-hoc test, $p < .05$). °, Different from training (Student's t test, $p < .05$).

observed, with differences between males and females in the first session ($t[42] = 2.865$, $P = .006$), as well as between males and females in proestrus in the first session ($F[4, 39] = 2.66$, $P = .047$). A statistically significant effect was also observed between the first and second sessions in proestrus females ($t[12] = 2.444$, $P = .031$). Figure 3 summarizes data for fecal boluses (Panel A: Sex differences in number of fecal boluses; Panel B: Estrous cycle effects on number of fecal boluses; Panel C: Difference in number of fecal boluses between training and test).

Discussion

The present data indicates that the estrous cycle influenced both parameters of immobility: the increases in the latency of the first immobility and the decreases in total immobility time by females in diestrus and proestrus. The effects of the estrous cycle on the number of fecal boluses, observed mainly in the first session, are conceivably indexical of a smaller threshold for stress in female rats, especially when in proestrus. The present results replicate the increase in the latency for the first immobility in diestrus and proestrus and the decrease in total immobility time in the first session by females in diestrus reported by Barros and Ferigolo (1998) and Marvan et al. (1996). In addition, they support data also obtained in two sessions with learned helplessness, another animal model of depression (Jenkins et al., 2000).

The present results support previous reports on the behavioral effects of the estrous cycle in other animal models. Diestrus has different effects in different models. In the elevated plus-maze, it causes increases in the open arm exploration (Diaz-Veliz et al., 1989, 1997; Gouveia Jr. & Morato, 2002); in the two-way avoidance task, it enhances acquisition (Diaz-Veliz et al., 1989); it increases long term food ingestion (Laviano et al., 1996) and improves palatability reactivity (Claker & Ossenkopp, 1998); and finally, in the elevated T-maze, it increases the first avoidance latency, or baseline (Gouveia Jr. et al., 2004).

Our data, however, failed to replicate the well-known increase in the amount of immobility observed between the first and the second sessions in males (e.g. Porsolt et al., 1977, 1978). The absence of this effect is possibly due to the small dimensions of our apparatus and to the height of the water column. Our apparatus, although very similar in dimensions to the one used by Porsolt et al. (1977, 1978) in their original papers, is smaller than those used by other authors in some more recent papers (Molina-Hernandez & Tellez-Alcantara, 2001; Cryan, Marlou & Lucki, 2002; Drossopoulou et al., 2004). In these studies, the investigators used larger cylinders and reported less immobility in the first session and larger increases in the second one.

The differences in total immobility in the training session between males and females in diestrus are conceivably generated by the action of progesterone. In general, there are peaks of progesterone secretion in two phases of the cycle, diestrus and estrus, but in diestrus it is secreted together with estradiol (Schwartz, 1969). Changes in progesterone metabolites in the hippocampus were observed in female rats in proestrus, modulating immobility in the FST (Frye & Walf, 2002). It is possible that the concomitant presence of both estradiol and progesterone tends to mutually cancel each other's effects on behavior (Bethea et al., 1999). Data in the elevated plus-maze support this hypothesis (Gouveia Jr. & Morato, 2002). Support also comes from other animal models, such as two-way avoidance (Beatty & Beatty, 1970), open-field (Blizand et al., 1975) and conditioned avoidance (Diaz-Veliz et al., 1989). In all these studies, several behavioral measures remained unchanged when the authors administered both hormones (each of which produces effects when given in isolation) simultaneously.

Estrogen modulates the firing activity of dorsal raphe nucleus 5-HT neurons in female rats (Robichaud & Debonnel, 2005), and induces changes in 5-HT1A binding and G-protein activation (Österlund, Halldin & Hurd, 2000; LeSaux & Di Paolo, 2005). A light-cycle-dependent effect on extracellular serotonin concentrations in the mediobasal hypothalamus was observed in females in diestrus and proestrus (Maswood et al., 1999). CA1 firing in response to Schaffer colateral stimulation was also greater in estrus and proestrus, while CA3 responses due to repetitive hilar stimulation evoked multiple population spiking in estrus and proestrus (Scharfman et al., 2003). Supposedly, those alterations, as well as changes in synaptic density, are a function of estrogenic activity in the median raphe nucleus (Prange-Kiel, Rune & Leranth, 2004). As such, the behavioral effects observed during proestrus could be correlated with the estrogen-progesterone equilibrium and its effects on the serotoninergic activity of the dorsal and median raphe nuclei, which, in turn, alters hypothalamic and hippocampal activity.

Progesterone effects are probably mediated by GABA, 5-HT1A and 5-HT2A receptors (Kaura et al, 2007); the GABA receptor is modulated by steroid hormones which can alter motor responses, on the one hand, and behaviors related to mood and anxiety, on the other (Biltran & Dowd, 1996; Concas et al., 1999; Fink et al., 1999). Studies have shown that, during estrus, 5-HT exhibits changes in synthesis, release, reuptake and catabolism (Biegon, Bercovitz & Samuel, 1980; Uphouse et al., 1986; Gundlah, Simon & Auerbach, 1998), and fluctuations in this indolamine are correlated with the action of estrogen and progesterone (Kueng, Wirz-Justice & Chappuis-Arndt, 1976; Di Paolo et al., 1983; Renner, Krey & Luine, 1987; Gereau, Kedzie & Renner, 1993; Famer et al., 1996). Some authors report variations in 5-HT extracellular concentrations in diestrus, the higher concentrations being found in the hypothalamus (Gundlah et al., 1998; Maswood et al., 1999).

Some reports have shown that progesterone may modulate the $GABA_A$ receptor function by potentiating it (Lambert et al., 2003; Lambert & Belelli, 2005; Kaura et al., in press) while inhibiting the activity of 5-HT neurons (Kaura et al., in press). Other studies on lordosis behavior reported that the activation of 5-HT1A and 5-HT2 receptors present opposite effects: 5-HT1A activation inhibits lordosis while 5-HT2 activation facilitates it (Wolf, Calderola-Patuszka & Uphouse, 1998; Uphouse et al., 2003). Stressing rats also causes lordosis to decrease, similar to the 5-HT1A receptor activation effects (Uphouse et al., 2003). In addition, 5-HT2A activation causes an antidepressant effect, but only in the presence of estrogen (Rybaczyk et al., 2005).

One shortcoming of the methodology used in this experiment is that estrous cycle stages were not analysed in the second session. Given that the FST seems to alter hypothalamic activity (Wotjak et al., 1998, 2001), it is possible that the stage of the estrous cycle in the second session was altered by the increase in estrogen (Shors et al., 1999); as such, our results would be more difficult to analyze, restraining our hypotheses to the observable effects on behavior. However, the observation that estrous cycle stages mediated emotional elements of the FST, but not its memory elements, is consistent with data found elsewhere (Palanza, 2001).

Based on the above discussion, a hypothesis can be made that the behavioral effects observed in the present experiment may be due to the combined effects of (1) a decrease in extracellular serotonin, (2) a decrease in 5-HT2 activity, (3) an increase in 5-HT1A activity, as well as (4) an increase in $GABA_A$ receptor activation. These effects are caused by changes in circulating levels of estrogen and free progesterone (a decrease in the first and an increase in the latter). The effect observed on

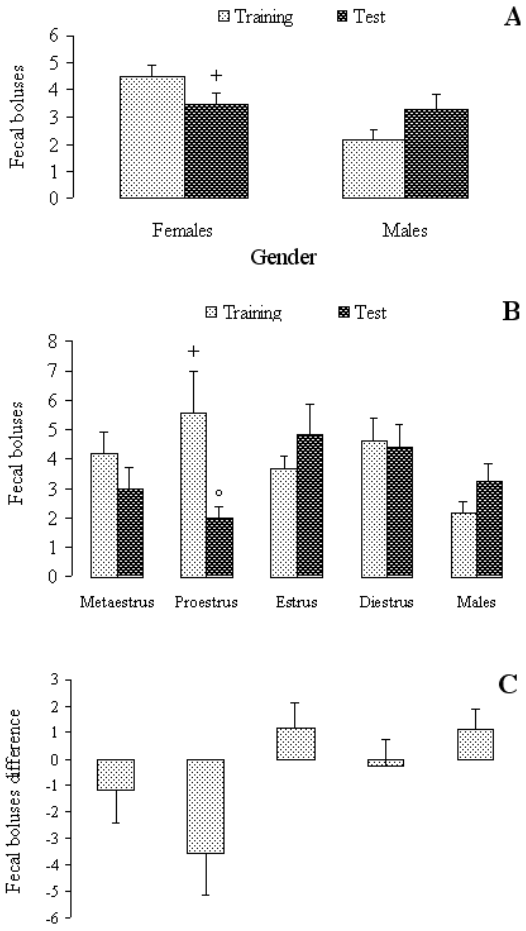

Figure 3. Number of fecal boluses (n, mean) by males and females in different phases of the estrous cycle. Panel A: Sex differences in number of fecal boluses; Panel B: Estrous cycle effects on number of fecal boluses; Panel C: Difference in number of fecal boluses between training and test.+, Different from females (Student's t test, P<.05); ++, different from proestrus (Duncan post-hoc test, p<.05). *, Different from training (Student's t test, p<.05).

total immobility, but not on latencies for immobility in the second session, could be due to a differential effect of both hormones on systems that mediate learning and emotionality in the forced swim test (Shors & Leuner, 2003). One alternative hypothesis, arisen from the data of the present experiment, contrasted with evidence in the literature, is that the acute swim stress raised estrogen levels in the brain, either directly (Shors et al., 1999) or indirectly, via augmentation of oxytocin and/or arginine-vasopressin levels (Wotjak et al., 1998, 2001). However, more experiments are needed in order to further confirm and refine those hypotheses.

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