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Landmark learning in a navigation task is not affected by the female rats' estrus cycle

Clara A. Rodríguez[*1], Raúl Aguilar[2], and V.D. Chamizo[1]

[1]*Universitat de Barcelona,* [2]*University of Málaga (Spain)*

In two experiments rats were required to escape from a circular pool by swimming to an invisible platform that was located in the same place relative to one configuration of two landmarks (X and Y). The two landmarks were placed relatively far and equidistant from the hidden platform. Training could be either on consecutive days (Experiment 1) or every fourth day (Experiment 2). Subsequent test trials, without the platform, revealed a preference for searching in the correct quadrant of the pool. In Experiment 1 such a test performance was identical in two groups of females, one tested with high hormonal levels (i.e., in the proestrus phase) and the second one tested with low hormonal levels (i.e., either in the estrus, metaestrus or diestrus phase); in addition, these two groups differed from a third group of male rats (i.e., males had a better performance than females). Experiment 2 replicated the females' previous results with a better procedure. The experiment compared the performance of two groups of female rats which were both trained and tested always in the same estrus phase, one group in the proestrus phase, and the second group in the estrus phase. The implication of these results is that the estrus cycle has little impact on the performance of female rats when landmark learning in a navigation task.

Many studies have shown a profound impairment on a variety of spatial tasks after lesions in the hippocampus (for example, Morris, Garrud, Rawlins, & O'Keefe, 1982; Pearce, Roberts, & Good, 1998; Sutherland, Whishaw, & Kolb, 1983). In the study by Morris et al. (1982), female Lister rats were trained to escape from a water maze by climbing onto a platform

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and the time to reach the platform was measured. The rats were divided into four groups according to the type of surgery: total hippocampal lesion, superficial cortical lesion, sham surgery and no surgery. For all rats, there were two kinds of tasks, one in which a submerged and hidden platform occupied a constant position in relation to the varied distal room cues, and the other in which the platform was visible, so that the rats did not need to learn about the platform's position in relation to the room cues (according to the authors, place and cue navigation tasks, respectively). The results revealed that the hippocampal lesion group showed a profound impairment in the place navigation task (i.e., with the hidden platform); and this effect disappeared when the visible platform was used. The remaining three groups learned to escape rapidly from the water in the two kinds of tasks. Morris et al. (1982) concluded that the performance of the task in which the rats have to learn about the location of a hidden platform in relation to distal cues is hippocampal-dependent but not the other kind of task in which the platform is visible, thus supporting the idea that the ability of navigation, which is essential for the survival of animals, depends critically on the integrity of this limbic structure (O'Keefe & Nadel, 1978 – although see Sherry & Healy (1998), and Good (2002) for critical reviews many years afterwards).

Spatial tasks have been considered hormonally dependent (for a demonstration in the radial maze, see Williams, Barnett, & Meck, 1990). Moreover, several studies (for example Woolley, 2007; Woolley & McEwen, 1992) have shown evidence of neurobiological and electrophysiological changes in the hippocampus during certain phases of the estrus cycle of female rats. The hormonal and reproductive cycle of female rats, called estrus cycle, lasts about four-five days and consists of four distinct phases: proestrus, estrus, metaestrus and diestrus. The characterization of each phase is based on the proportion among three types of cells observed in a vaginal smear: epithelial cells, cornified cells and leukocytes. A proestrus smear consists of a predominance of nucleated epithelial cells; an estrus smear primarily shows anucleated cornified cells; a metaestrus smear contains the same proportion among leukocytes, cornified, and nucleated epithelial cells; and a diestrus smear primarily consists of a predominance of leukocytes. These different phases of the estrus cycle correlate with different levels of the sex hormone estradiol circulating. Estradiol levels begin to increase at metaestrus, reaching peak levels during proestrus and return to baseline at estrus. According to Woolley and McEwen (1992), during the phase of the estrus cycle in which occurs the peak level of estradiol (i.e., the proestrus phase), the hippocampus shows an increase in synaptic density in the apical cells of the

pyramidal cells of the CA1 area of up to 30%. These changes have been suggested to affect spatial performance. But the literature is inconsistent in their direction. Let's see a few examples.

In a study by Warren and Juraska (1997), two groups of synchronized female rats were trained and tested in a single day in a similar place navigation task like the one used by Morris et al. (1982) when the platform was hidden. One group was in the proestrus phase and the second one in the estrus phase. The results showed that although the groups did not differ when trained to reach the hidden platform, they differed on the test trial without the platform, where female rats in the estrus phase outperformed females in the proestrus phase (and for a similar result, see Markus & Zecevic, 1997). In a related study by Berry, McMahan, and Gallagher (1997), on a final test day in which two groups of females were synchronized (one in the proestrus phase and the second one in the estrus phase), the groups did not differ neither on the initial escape trials of the test day nor on the final test trial without the platform (and for similar results, see Harrel, Pleagler, Parson, Litersky & Barlow, 1993; Singh, Meyer, Millard & Simpkins, 1994). Finally, in a study by Healy, Braham, and Braithwaite (1999), also in the water-maze, differences in the performance of two groups of female rats were found on the final day of acquisition in which the two groups were synchronized (one group in the estrus phase and the second one in the proestrus phase). But the results found were exactly opposite to what could be expected according to Warren and Juraska (1997): females in the proestrus phase reached the platform faster than females in the estrus phase (and for similar results, see Frye, 1995). How can this be?

There are important procedural differences in the previous studies that could explain, at least partly, such discordant results. For example, the measure used (like time to reach the platform on escape trials, or time in the platform quadrant on test trials without the platform), or the presence or absence of curtains surrounding the pool that could prevent or not learning about some distal room cues (for further explanation, see the General Discussion section). Given this situation, the main aim of the present study was to conduct experiments to specifically see whether landmark learning is affected by the female's estrus cycle. In order to answer this question, circular black curtains surrounded the pool, with two three-dimensional landmarks inside this enclosure, so that no other room cues (like the shape of the room) could provide additional information to find the platform. The landmarks were hung from a false ceiling and rotated from trial to trial with the platform, thus preserving a constant relation between the platform and the landmarks (i.e., eliminating olfactory, auditory, and directional cues

outside the curtains). Four starting points were used. During acquisition, the rats were required to escape from the pool by swimming to an invisible platform that was located in the same place relative to one configuration formed by the two landmarks which were placed relatively far and equidistant from the hidden platform (as shown in Figure 1). After training the rats were tested, without the platform, in the presence of the landmarks, with the pool surface spatially divided into four quadrants: where the platform should have been, right to it, left to it and opposite to it. The time the rats spent in all the quadrants was measured.

The aim of Experiment 1 was to examine whether female rats tested in the proestrus phase (i.e., with high levels of estradiol, Group Proestrus) performed in the pool differently than females that were in a phase of the estrus cycle of low hormonal levels (specifically, either in the estrus, metaestrus or diestrus phase, all rats included under the name Group Others); this experiment also contained a third group of male rats. Then Experiment 2, with a better procedure, consisted of two groups of female rats which were both trained and tested in the same estrus phase (i.e., every fourth day). One group was always in the proestrus phase, Group Proestrus, and the second group always in the estrus phase, Group Estrus.

EXPERIMENT 1

Unpublished pilot work in our laboratory suggests that females with low hormonal levels (i.e., either in the estrus, metaestrus, or diestrus phases) do not differ in their performance when landmark learning. Following these results, in Experiment 1 a group of female rats was tested with low hormonal levels (i.e., either in the estrus, metaestrus or diestrus phase, all rats included under the name Group Others), a second group of females was tested in the proestrus phase (Group Proestrus), and a third group of animals were male rats (Group Males). During acquisition, an invisible platform was located in the same place relative to one configuration of two landmarks (X and Y), which were placed relatively far and equidistant from the hidden platform, as shown in Figure 1. After acquisition the two groups of females were synchronized and a test trial, without the platform, measured the preference for searching by the three groups in the four quadrants of the pool. Considering the conflicting evidence in the literature clear predictions could not be formulated in this experiment.

METHOD

Subjects. The subjects were 36 Long Evans rats, 12 males (Group Males) and 24 females, approximately five months old at the beginning of the experiment that had previously participated in a plus-maze experiment. The 24 females were divided into two differentiated groups of 12 according to the phases of the estrus cycle: the Group Proestrus, with high-hormonal level, and the Group Others, with low-hormonal level. The animals were housed in pairs of the same sex in standard cages, 25 x 15 x 50-cm, maintained on *ad lib* food and water, in a colony room with 12:12-hr light-dark cycle. The experiment took place within the first 8 hrs of the light cycle.

Determination of the estrus cycle. The rats were examined to establish the estrus cycle by a daily collection of vaginal smear for 8 days before the start of the experiment. In order to establish two distinct synchronized groups of females according to the different phases of the estrus cycle, the "Whitten Effect" (Whitten, 1966) was carried out which produces the synchronization of estrus in females by the exposure to male pheromones. Specifically, some shavings soaked in urine and feces of male rats were introduced in half of the cages of the female rats before the pretraining phase. During the experiment, they continued to be examined every day. On the test day, rats were examined both pre – and post-testing to ensure that they did not change over to the next estrus cycle phase during testing. We performed vaginal examination following the procedure used by Marcondes, Bianchi and Tanno (2002): the females were raised her tail gently inserting a cotton swab, previously soaked in saline, into the vagina to obtain the cytology by circular movements. The product of this cytology was examined under a light microscope (10x objective) to determine the phase of the estrus cycle in which each animal was, following the procedures used in Sava and Markus (2005) and Feder (1981): the proestrus was defined as a predominance of epithelial or nucleated cells, the estrus as a predominance of cells without nuclei, or cells cornified, the metaestrus as a combination of cornified cells and leukocytes and diestrus as a predominance of leukocytes. The rats were divided into two groups: Group Proestrus if 50-70% of visible cells were nucleated (i.e., epithelial), and Group Others if nucleated cells were less than 15%. Furthermore, to minimize the effects that the manipulation described above might result in females, males received a similar handling: they were turned upside down to expose the perineal region, and then the scrotum was wiped with a cotton swab.

Apparatus. The apparatus was a circular swimming pool, made of plastic and fibre glass, modelled after that used by Morris (1981). It measured 1.58-m in diameter and 0.65 m deep, and was filled to a depth of 0.49-m with water rendered opaque by the addition of 1cl/1 of latex. The water temperature was maintained at 22±1 ºC. The pool was situated in the middle of a large room, mounted on a wooden platform 0.43-m above the floor, and surrounded by black curtains reaching from the ceiling to the base of the pool and forming a circular enclosure 2.4-m in diameter. Two objects or landmarks were placed inside the enclosure, suspended from the false ceiling, 0.23-m above the surface of the water and with the mid-line directly above the wall of the pool. In order to ensure that the rats used these landmarks, rather than any inadvertently remaining static room cues, to locate the platform, between each trial the landmarks and platform were semi-randomly rotated with respect to the room (90º, 180º, 270º, 360º), with the restriction that all parts of the room were used equally each day. A closed-circuit video camera with a wide-angle lens was mounted 1.75-m above the centre of the pool inside the false ceiling, and its picture was relayed to recording equipment in an adjacent room. A circular platform, 0.11-m in diameter and made of transparent Perspex, was mounted on a rod and base, and could be placed in one quadrant of the pool, 0.38-m from the side, with its top 1-cm below the surface of the water, as shown in Figure 1. The two landmarks used were as follows: landmark X was a 30-cm diameter plastic beach ball with alternative blue, white, yellow, white, red, and white vertical segments; and landmark Y was a 28-cm cube with a black line in the centre of each side; both of them were approximately 110-cm from the hidden platform.

Procedure. There were three types of trials: pretraining, training, and test trials. Pretraining consisted of placing a rat into the pool without landmarks but with the platform present. The rat was given 120-s to find the platform, and once the rat had found it, it was allowed to stay on it for 30-s. If a rat had not found the platform within the 120-s, it was picked up, placed on it, and left there for 30-s. The platform was moved from one trial to the next, and the rat was placed in the pool in a different location on each trial (at I, II, III, IV, in Figure 1), as far as possible equally often on the same or opposite side of the pool from the platform and with the platform to the right or to the left of where the rat was placed. Rats were given five such pretraining trials over 2 days, with two trials on Day 1, and three on Day 2. The animals were run in squads of eight and spent the intertrial interval (ITI) in small individual compartments.

The procedure for training was similar to that of pretraining except that the two landmarks, X and Y, were always present on each trial. Rats were given eight trials per day over seven consecutive days (a total of 56 trials). These trials had an inter-trial interval (ITI) of 8-10-min, and the platform and the landmarks were rotated between trials, with the platform always maintaining a fixed position in relation to the two landmarks, as shown in Figure 1.

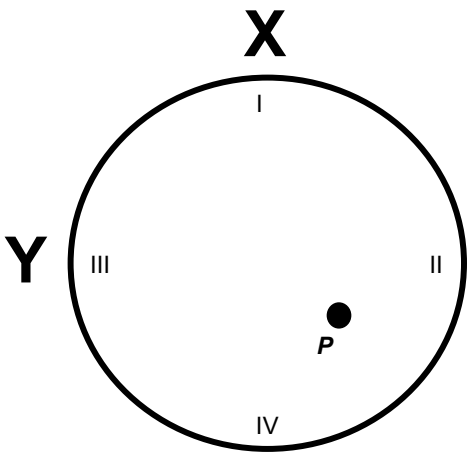

Figure 1. A schematic representation of the pool and the landmarks used (X and Y), as well as the platform (P) and the starting points (I, II, III, IV).

Following training, all rats received a test day consisting of eight retraining escape trials, followed by a single test trial. Escape trials were as in training. The test trial consisted of placing the rat into the pool, with landmarks present but without the platform, and leaving it there for 60-s. The same four starting positions were used as in training. For purposes of recording the rat's behavior, on test trials the pool was spatially divided into four quadrants: were the platform should have been, right to it, left to it and opposite to it. The amount of time the rat spent in all the quadrants was recorded.

An alpha level of 0.05 was adopted for all statistical analyses. Only significant results are presented.

RESULTS AND DISCUSSION

Latencies to find the platform decreased over the course of the 5 initial pretraining trials: in males from a mean of 70.33-s on Trial 1 to a mean of 31.50-s on Trial 5; in females of Group Proestrus from a mean of 94.59-s on Trial 1 to a mean of 64.83-s on Trial 5, and in females of Group Others from a mean of 117.25-s on Trial 1 to a mean of 47.42-s on Trial 5. An ANOVA conducted on these data, taking into account the variables group (Group Males, Group Proestrus, Group Others) and trials (1 to 5), showed that the only significant variable was trials, $F(4,132) = 7.60$. Neither the variable group nor the interaction group x trials were significant (Fs < 3.0). All rats improved their performance as pretraining progressed. This suggests that females are not more likely than males to spend time exploring the pool rather than swimming directly to the platform (for the same result, see Forcano, Santamaría, Mackintosh & Chamizo, 2009, and Rodríguez, Torres, Mackintosh & Chamizo, 2010).

Latencies to find the platform also decreased over the course of the training days (Figure 2). An ANOVA conducted on these data, taking into account the variables group (Group Males, Group Proestrus, Group Others) and days (1 to 7), showed that the variables group and days were significant, as well as the interaction, $F(2,33) = 16.15$, $F(6,198) = 45.59$ and $F(12,198) = 2.07$, respectively. Further analysis of the interaction group x days, simple main effects, showed that the groups differed on days 1, 2, 4-7, $F(2,33) = 6.29$, 3.46, 11.45, 7.69, 53.78, 8.79, respectively. Subsequent pair comparison (Newman-Keuls) revealed that on days 1 and 2 only the comparison between Group Males and Group Proestrus was significant (ps<.05); on days 4 and 5, Group Males differed from both Group Proestrus and Group Others (ps<.05), which did not differ from each other, and on days 6 and 7 Group Proestrus differed from both Group Males and Group Others (ps<.05), which did not differ from each other. Thus, in general, Group Males reached the platform faster than the female groups, Group Proestrus and Group Others, with some suggestion that Group Others reached the platform faster than Group Proestrus[1]. An ANOVA of the escape trials during the test day (day 8), showed that the three groups did not differ ($F < 1$).

[1] One explanation in relation to day 6 of acquisition refers to the rats' weekly bedding change, which is conducted by experts different from the experimenters, and that significantly alters the rats. It is possible that the experimental session of the rats in Group Proestrus took place immediately afterwards such a change, while that was not the case in the rats belonging to the other groups. Steps were taken to avoid this potential problem in Experiment 2.

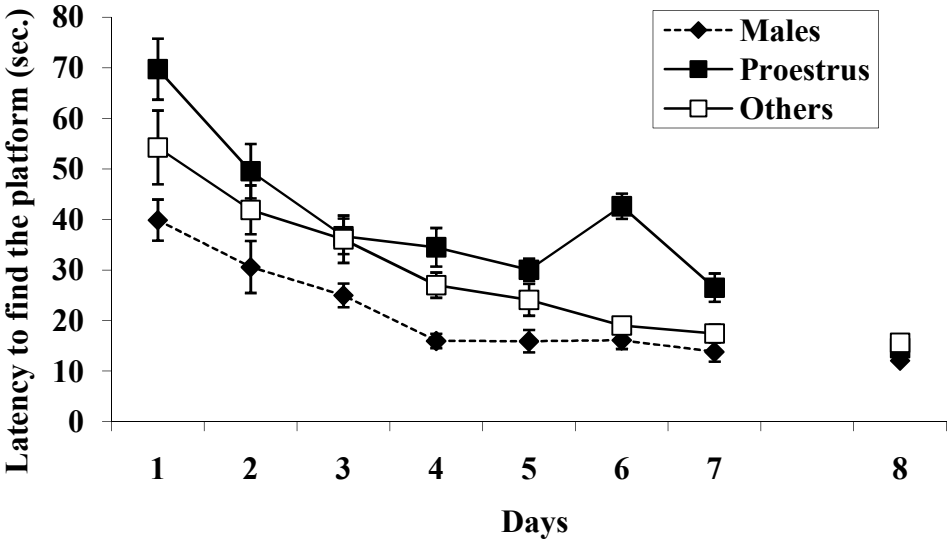

Figure 2. Mean escape latencies for the three groups of Experiment 1 during the training phase and the test day. Error bars denote standard error of the means.

Figure 3 shows the time spent in the four quadrants (i.e., the training quadrant, right to it, left to it and opposite to it –TQ, RQ, LQ, and OQ, respectively) by the three groups on the test trial and also a small asterisk above each bar indicates whether the rats's performance differed significantly from chance (i.e., 15 sec). Student *t* tests were used in each group (Group Males, Group Proestrus and Group Others) to compare rat's performance in the TQ, the training quadrant, with the performance in the other three quadrants (RQ, LQ, OQ) in order to evaluate preferences for the training quadrant, TQ. The three comparisons in Group Males differed significantly [$t(11)$ = 8.75, 8.34, and 11.57 –TQ vs RQ, LQ, and OQ, respectively]. The same was true in Group Proestrus [$t(11)$ = 2.55, 2.88, and 4.57 –TQ vs RQ, LQ, and OQ, respectively], as well as in Group Others [$t(7)$ = 2.15, 2.34, and 4.72 –TQ vs RQ, LQ, and OQ, respectively]. Then, an ANOVA conducted of the time spent in each of the four quadrants (TQ, RQ, LQ, and OQ) by each of the three groups (Group Males, Group Proestrus, and Group Others), showed that the variable quadrant, as well as the interaction group x quadrant were significant, $F(3,99)$ = 50.39 and $F(6,99)$ = 3.98, respectively, even when the interaction term degrees of freedom were reduced, in accord with the fact that each rat's scores for the

four quadrants summed to 60 sec. Further analysis of the interaction group x quadrant, simple main effects, revealed that the groups differred both on the training and the right quadrants (TQ and RQ), $F(2,33) = 4.71$, and 3.37, respectively. Subsequent Newman-Keuls comparisons showed that, both in the TQ and in the RQ quadrants, Group Males differed from both Group Proestrus and Group Others ($ps < .05$), which did not differ from each other ($p > .05$).

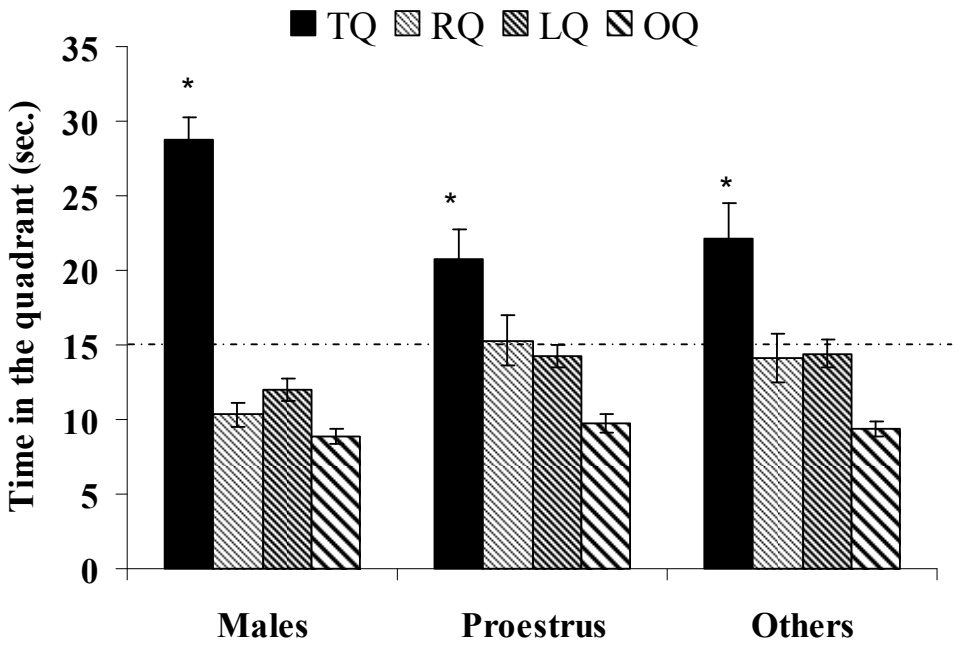

Figure 3. Mean time spent in the four quadrants [i.e., the training quadrant (where the platform should have been), right to it, left to it and opposite to it –TQ, RQ, LQ, and OQ, respectively] by the three groups during the test trial of Experiment 1. A small asterisk above each bar indicates whether each group differed significantly from chance. Error bars denote standard error of the mean.

The main results of this experiment are as follow. On the test day, both during the escape trials and on the test trial, there was no difference between females with high hormonal levels (i.e., in the proestrus phase) and females with low hormonal levels (i.e., with rats in the estrus, metaestrus, and diestrus phases combined). In addition, male rats outperformed the two groups of females on the test trial. Unfortunately, the results were not so clear during the escape trials of the acquisition phase. Only on days 1-5 of this phase males reached the platform faster than females, which did not differ between them. It could be argued that one problem in this experiment that could explain, at least partly, the lack of differences on the test day between the female groups, could be the fact that in Group Others the animals were in different phases of the estrus cycle and this could have biased the results of this group because the levels of estradiol vary across the estrus, metaestrus, and diestrus phases (see Healy et al., 1999). In fact, considering the literature (for example Berry et al., 1997; Healy et al., 1999; Warren & Juraska, 1997), the standard manipulation is to compare two groups of female rats, one in the proestrus phase and the other in the estrus phase. Therefore, perhaps the procedure in the present experiment conducted with the female rats of Group Others could have been a problem.

EXPERIMENT 2

The aim of Experiment 2 was to solve the problems mentioned in Experiment 1 by improving the general procedure by means of two specific manipulations. Firstly, the experiment consisted of two groups of female rats that were trained and tested only in the days in which they were at a specific phase of the estrus cycle (i.e., every fourth day for Long Evans rats –instead of training on consecutive days as in Experiment 1), thus solving the possible bias produced by training the rats in consecutive days, while changing the estrus phase day by day. Secondly, one group was trained and tested with high hormonal levels (i.e., in the proestrus phase, Group Proestrus –as in Experiment 1), and the second group with low hormonal levels (although in the estrus phase only, Group Estrus –instead of combining rats in the estrus, metaestrus, and diestrus phases, as in Experiment 1). These manipulations in comparison to Experiment 1 were introduced in an attempt to facilitate, as much as possible, the influence of the different phases of the estrus cycle (proestrus *vs.* estrus) on female rats' navigation. Thereby, if the two groups of females do not differ even under such favorable conditions, then this result would support the idea that the estrus cycle has less importance in landmark learning than previously thought.

METHOD

Subjects and apparatus. The subjects were 16 female Long Evans rats, approximately five months old at the beginning of the experiment that had previously participated in a taste-aversion experiment. The animals were divided into two differentiated groups of 8 according to the phases of the estrus cycle: the Group Proestrus, with high-hormonal level, and the Group Estrus, with low-hormonal level. The animals were kept and maintained as in the previous experiment. The apparatus, the experimental room, and the landmarks were also the same as those used in Experiment 1.

Determination of the estrus cycle. In this experiment, rats were examined to establish the estrus cycle by a daily collection of vaginal smear for 8 days before the start of the experiment. During the experiment, rats were also examined daily. The procedure of determination of the estrus cycle was identical as that used in Experiment 1.

Procedure. The general procedure was the same as in Experiment 1, although with one important exception. For pretraining, training and the test trial the experiment was carried out every four days, so that the animals were always on the same hormonal level each day on which the experimental session took place (high hormonal levels for Group Proestrus and low hormonal levels for Group Estrus).

RESULTS AND DISCUSSION

Latencies to find the platform decreased over the course of the 5 initial pretraining trials: in Group Proestrus from a mean of 117.1-s on Trial 1 to a mean of 33.2-s on Trial 5, and in Group Estrus from a mean of 114.0-s on Trial 1 to a mean of 34.3-s on Trial 5. An ANOVA conducted on these data, taking into account the variables group (Proestrus, Estrus) and trials (1 to 5), showed that the only significant variable was trials, $F(4,54) = 16.89$. Neither the variable groups nor the interaction groups x trials was significant (Fs < 0.5). All rats improved their performance as pretraining trials progressed.

Latencies to find the platform also decreased over the course of the training days (see Figure 4). An ANOVA conducted on these data, taking into account the variables group (Proestrus, Estrus) and days (1 to 7), showed that the only significant variable was days, $F(6,84) = 38.68$. Neither the variable groups nor the interaction groups x days was significant (Fs < 0.5). All rats improved their performance as training progressed. An

ANOVA of the escape trials during the test day (day 8), showed that the groups did not differ (Fs < 2.5).

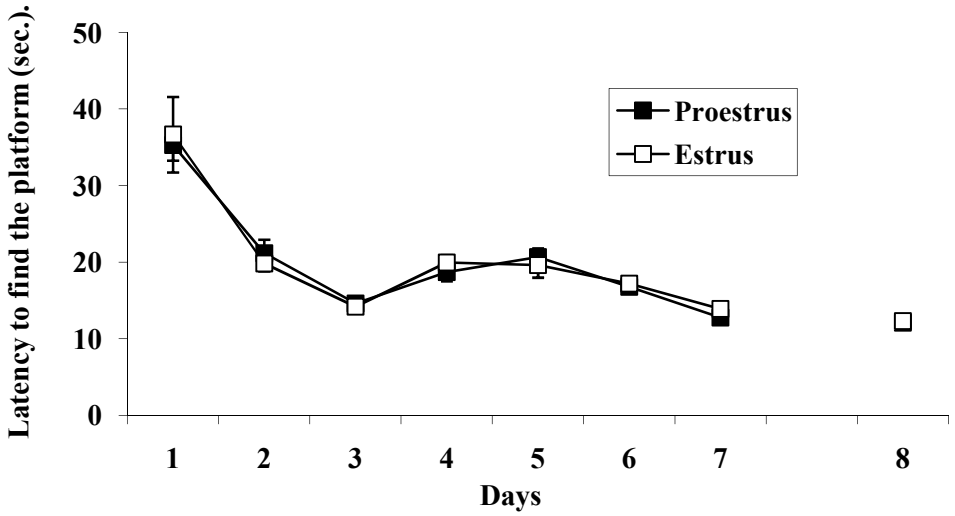

Figure 4. Mean escape latencies for the two groups of Experiment 2 during the training phase and the test day. Error bars denote standard error of the means.

Figure 5A shows the percentages of epithelial or nucleated cells, cornified cells, leukocytes, and time in TQ, the training quadrant (i.e., where the platform should have been), by the rats in the different phases of the estrus cycle during the test trial. The results of a Pearson correlation analyses (see Figure 5B) revealed no differences between the number of epithelial cells and the amount of time spent in TQ, the training quadrant, rs = 0.11. Moreover, the R^2 statistic was also used (Field, 2009) to explain the amount of variation in the percentage of time spent in TQ by the different rats as a function of the amount of epithelial cells, $R^2 = 0.01$. These results show a lack of differences between the rats in the two phases of the estrus cycle (i.e., proestrus and estrus).

A

Experiment 2 Test 1	% Epithelial cells	% Cornified cells	% Leukocytes	% Time in TQ
Proestrus (n=8)	90.63	0.00	5.31	48.59
Estrus (n=8)	0.00	78.44	0.00	47.77

B

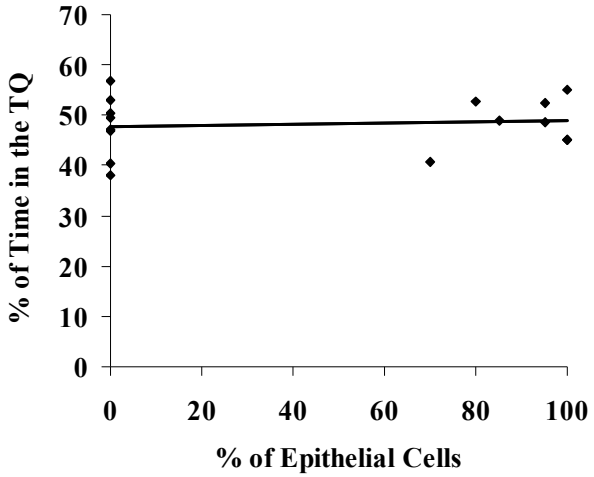

Figure 5. A: Mean percentages of epithelial or nucleated cells, cornified cells, leukocytes, and time in TQ, the training quadrant (i.e., where the platform should have been), by the rats in the different phases of the estrus cycle during the test trial of the Experiment 2. B: Correlation, with the linear regression model line, between the percentages of time spent in the training quadrant (TQ) and the amount of epithelial cells for all the rats (n= 16) of Experiment 2.

Figure 6 shows the time spent in the four quadrants (i.e., the training quadrant, right to it, left to it and opposite to it –TQ, RQ, LQ, and OQ, respectively) by the two groups on the test trial, and also a small asterisk above each bar indicates whether the rats's performance differed

significantly from chance (i.e., 15 sec). Student *t* tests were used in each group (Group Proestrus and Group Estrus) to compare rat's performance in the TQ, where the platform should have been, with the performance in the other three quadrants (RQ, LQ, OQ) in order to evaluate rats' preferences for the training quadrant, TQ. The three comparisons in Group Proestrus differed significantly [$t(7) = 9.53$, 10.59, and 27.63 –TQ vs RQ, LQ, and OQ, respectively]. The same was true in Group Estrus [$t(7) = 7.61$, 9.92, and 13.64 –TQ vs RQ, LQ, and OQ, respectively]. Then, an ANOVA conducted of the time spent in each of the four quadrants (TQ, RQ, LQ, and OQ) by each of the two groups (Group Proestrus and Group Others), showed that the variable quadrant was significant only $F(3,42) = 168.31$. Neither the variable group nor the interaction groups x quadrants were significant (Fs < 0.5).

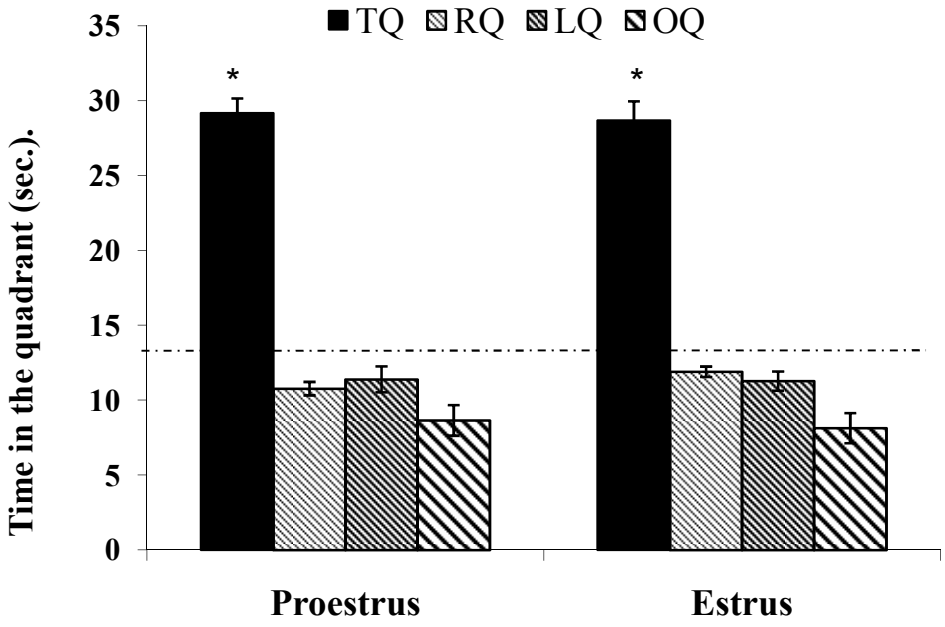

Figure 6. Mean time spent in the four quadrants [i.e., the training quadrant (where the platform should have been), right to it, left to it and opposite to it –TQ, RQ, LQ, and OQ, respectively] by the two groups of Experiment 2 during the test trial. A small asterisk above each bar indicates whether each group differed significantly from chance. Error bars denote standard error of the mean.

On both the escape and the test trials, the results of this experiment showed that the rats' performance (i.e., females with high hormonal levels and females with low hormonal levels) did not differ in the presence of the two landmarks, thus confirming, once resolved the potential biases, the results obtained in Experiment 1.

GENERAL DISCUSSION

The present experiments have consistently shown that the estrus cycle does not influence the female rats' performance in a highly controlled navigation task based on two landmarks which were placed some distance away from a hidden platform, a hippocampal-dependent task (Morris et al., 1982; Pearce et al., 1998; Sutherland et al., 1983). The results of Experiment 1 revealed that there was no difference between females with high hormonal levels and females with low hormonal levels. This experiment also showed that both in training and on the test trial, males had a better performance than the two female groups. Thus, although all rats showed spatial learning, as the test trial revealed, performance was better in Group Males than in Group Proestrus and Group Others, which did not differ between them. This result implies that the males' performance under our specific conditions is better than the females' performance, independently of their hormonal levels. This sex difference is a result often found in the literature (for a meta-analysis in the Morris pool see Jonasson, 2005).

Experiment 2, where a better procedure was used, confirmed the female results obtained in Experiment 1. In Experiment 2 the rats were only trained and then tested on the days in which they were with high hormonal levels (i.e., in the proestrus phase, Group Proestrus) or with low hormonal levels (i.e., in the estrus phase, Group Estrus) and no difference between the two groups appeared, neither in the acquisition phase nor in the test day. These results give support to the females' performance in Experiment 1, while showing that hormonal fluctuations related to the estrus cycle do not influence a hippocampal-dependent task when landmark learning. The implication is that if a female rat learns only in those days in which the increase in synaptic activity in the hippocampus due to the high hormonal levels occurs, the performance of this animal in a navigation task with a hidden platform will not be better or worse than the performance of another female rat that learns the same task but only in the days in which its hormonal levels are low.

As these findings fit some prior studies (Berry et al., 1997; Harrels et al., 1993) but not others (Healy et al., 1999; Warren & Juraska, 1999), we wondered how that could be. It should be noted that the previous studies have used different procedures, so it should not be surprising that they also obtained different outcomes. We believe that a crucial procedural difference among these studies (Berry et al., 1997; Healy et al., 1999; Warren & Juraska, 1999) in order to explain their discordant results refers to the use (or not) of curtains surrounding the pool, thus preventing (or not) the rats learning about other sources of information outside the curtains. While both Berry et al. (1997) and the present experiments used curtains surrounding the pool, neither Warren and Juraska (1997) nor Healy et al. (1999), used such curtains. In the first case the results of the two studies coincide (although Berry et al. worked with an unspecified number of panels inside this enclosure) while in the second case they do not. A main question to answer is: How do rats learn to locate an invisible platform in a water maze when the platform maintains a fixed position with respect to distal information? Since Morris's (1981) seminal work (see also Morris et al., 1982), there is a rather general consensus that navigation in the circular pool when the platform is invisible involves learning its location based on its fixed spatial relationship to a number of distal landmarks (such as pieces of furniture, windows, curtains, lamps… etc). But at present we know that other distal alternatives are also possible: the geometry or shape of the room (Williams et al., 1990), directional room cues (Hamilton, Akers, Johnson, Rice, Candelaria, Sutherland, Weisend, & Redhead, 2008), the geometry or shape of the apparatus (Pearce et al., 2006), the wall of the pool (Hamilton, Johnson, Redhead, & Verney, 2009), one object located immediately around the circumference of the pool (Chamizo, Rodrigo, Peris, & Grau, 2006), a small set of objects located immediately around the circumference of the pool (Prados & Trobalon, 1998; Rodrigo, Chamizo, McLaren & Mackintosh, 1997; Roof & Stein, 1999) and other features of the room, like static directional cues (such as constant noise from lighting or pipe noises) which perhaps are not perceived by the experimenters. Moreover, in many experiments, several of these different types of cues may be simultaneously available for use by the rat. Under which conditions are they learned in parallel and when do the different sources of information interact remains an open question. According to the results obtained by Berry et al., (1997) and those presented here, it seems that the estrus cycle has little impact on the rats' performance in the Morris pool when the location of the hidden platform is defined by landmarks, being excluded other sources of information (for example the geometry or shape of the room and directional visual room cues) by the use of curtains surrounding the pool and multiple

starting positions. But does the estrus cycle influence the rats' performance when these cues can be learned? We do know that when rats are trained in a triangular-shaped pool to find a hidden platform, whose location was defined in terms of two sources of information, one landmark outside the pool and one particular corner of the pool, the estrous cycle of females (proestrus *vs*. metaestrus phases) did not influence their performance (Rodríguez et al., 2010, Experiment 1). Female rats did not differ neither on the escape trials of the test day nor on the final test trial (where all of them showed a clear preference for the landmark).

What would have happened if the rats in the Healy et al. (1999) study would have had a final test trial, without the platform, like in the study by Warren and Juraska (1997)? Because stress and frustration plays a role both in learning and in performance (for a review see Shors, 2004), we believe that the amount of stress and frustration must be higher when an animal is tested without the platform during a whole minute than when it is required to reach the platform (i.e., to swim to a safe place for a few seconds). Another crucial aspect between these two studies (Healy et al., 1999 and Warren & Juraska, 1997) refers to the complexity of the task. In the study by Healy et al. (1999), the animals were required to find a hidden platform and received four daily trials for twenty days. For the four trials of each session the landmarks and the hidden platform remained in the same place, but the platform position varied every session in relation to the cues provided by the experimental room. Thus, the rats had to learn, based on the cues provided by the experimental room, a new platform position every day (for a related procedure, see Pearce et al., 1998). This task seems rather more complex than that conducted by Warren and Juraska (1997), in which the animals were required to find a hidden platform, whose position was always kept constant in relation to the cues provided by the experimental room, and received 16 trials in a single day. Probably, the discordant results of these two studies (Healy et al., 1999; Warren & Juraska, 1997), could be understood considering the differential amount of stress, frustration, and complexity of the tasks as main factors.

Different experiments (Roof & Stein, 1999; Forcano et al., 2009) have shown that males outperform females only when the task is relatively complex. Equally important is the fact that some authors have found that females with high levels of estradiol tend to perform as males do (Williams et al., 1990). Consequently, it could be reasonable to expect that, when the task is difficult, as in the study of Healy et al. (1999), females with high hormonal levels outperform females with low hormonal levels. This facilitatory effect observed in females with high hormonal levels should disappear with an easier task, as in fact happened in the study by Warren

and Juraska (1997). But admittedly, it is far from clear why females with low hormonal levels could perform the 60-s test trial significantly better than females with high hormonal levels (i.e., the specific results by Warren & Juraska, 1997). We believe that the many differences in the procedures used by the studies reviewed makes it very difficult to compare them.

In conclusion, the present study shows that hormonally-dependent variations in hippocampal functioning associated with different phases of the estrus cycle do not affect the rats' performance in a landmark learning navigation task, thus replicating, with a more controlled procedure (i.e., by means of two three-dimensional landmarks, specifying their main characteristics and the position both between them and with regard to the platform), the results by Berry et al. (1997). The major finding of the present manuscript is that we have clearly demonstrated that landmark learning, at least when it is based on two relatively distal and equidistant from the platform's position landmarks, is not affected by the female estrus cycle. Finally, the present results may be also interpreted as an encouragement to further explore the factors that might affect the learning of hippocampal-dependent tasks when multiple sources of spatial information are available (for example, room-shape learning when curtains are not used).

RESUMEN

El aprendizaje en base a puntos de referencia en una tarea de navegación no se ve afectado por el ciclo estral de la rata. En dos experimentos en piscina circular se entrenó a unas ratas a encontrar una plataforma invisible que estaba localizada siempre en el mismo lugar en relación a una configuración de dos puntos de referencia (X e Y), que se encontraban relativamente lejos y equidistantes de la plataforma. El entrenamiento se llevó a cabo durante días consecutivos (Experimento 1) o cada 4 días (Experimento 2). Ensayos de prueba posteriores, sin la plataforma, mostraron que las ratas preferían buscar en el cuadrante correcto de la piscina. En el Experimento 1 la ejecución en el ensayo de prueba fue idéntica en dos grupos de hembras, uno puesto a prueba con altos niveles hormonales (es decir, en la fase de proestro) y el otro con bajos niveles hormonales (concretamente, en la fase de estro, metaestro o diestro); además, ambos grupos de hembras difirieron de un tercer grupo de machos (los machos ejecutaron mejor la tarea que las hembras). El Experimento 2 replicó los datos anteriores obtenidos por las hembras, con un procedimiento mejorado. El experimento comparó la ejecución de dos grupos de hembras que fueron entrenados y puestos a prueba siempre en la misma fase del ciclo estral, un grupo en la fase de proestro y el segundo en la fase de estro. La implicación de estos resultados es que el ciclo estral tiene muy poco impacto en el aprendizaje basado en puntos de referencia en una tarea de navegación espacial.

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