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Effects of waiting time between trials and water temperature on cognitive functions, body temperature and body weight in rats in Morris water maze

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ABSTRACT

The Morris water maze (MWM) is a widely used test among neurobiologists to measure spatial memory. The implementation of this test carries the risk of hypothermia periods in animals. The level of hypothermia may affect age-related memory processes as a significant factor. The occurrence of hypothermia throughout the MWM protocol should be better understood as hypothermia may impair memory performance. Ensuring the standardization of the experiments and minimizing side effects require a detailed examination of the hypothermia-related processes. Our study aims to replicate and extend the data of previous studies in terms of determining the possible species-specific variations and provide data for reorganizing the time intervals. In this study, rats (Wistar Hannover) were used and grouped according to the differences in the inter-trial interval (ITI) (30-s and 13-min) and water temperatures (20 °C and 24 °C). The effects of ITI and water temperature on probe performance were analysed statistically (mixed two-way ANOVA). Results showed that the 13 minute waiting group of animals performed statistically better in the MWM probe phase compared to the 30 second waiting group. The prolongation of ITI between the tests was found to have a positive impact on the memory performance. Longer ITI should be preferred instead of the frequently used 30-60 second test intervals. Thus, animals will be exposed to less stress and more reliable results can be obtained, also possible side effects of hypothermia can be minimized while performing the MWM test.

Keywords: comorbidity, hypothermia, memory, spatial, probe performance

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Introduction

The Morris water maze (MWM) test is one of the most preferred cognitive tests in the field of age-related learning and memory loss (Ecevitoglu et al., 2020; Pezük et al., 2006; Iivonen et al., 2003; Morris, 1984). It is also a reliable spatial learning and reference memory test and is widely used in neurobiological studies. However, this test has strengths and weaknesses that arise from various causes. During the administration protocol of the MWM test on the animals, different levels of hypothermia can occur when animals remain in the water for certain periods. Iivonen (2003) has performed different MWM protocols and reported that female mice which were exposed to short inter-trial interval (ITI) and the lower water temperature had up to 9 centigrade lower body temperature. Therefore, to ensure the standardization and reliability of the experiments and to minimize side effects for the animal welfare a detailed examination

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of the hypothermia-related process of the test and its optimization according to certain criteria is required (age, sex, body weight, disease model, etc.). Especially in experimental studies where variables such as stress level, body temperature, and body weight need to be stable, standard protocols can be re-adjusted in a species-specific manner.

Studies in both mice and rats have reported that hypersensitivity to stress negatively affects the performance on the MWM test (D'Hooge & De Deyn, 2001). Additionally, studies have shown that extending the ITI eliminates the "net cooling effect of water" which causes a drop in body temperature. (Iivonen et al., 2003). It has been reported that this effect may also be age-related (Iivonen et al., 2003; Lindner & Gribkoff, 1991; Hamm, 1981). Therefore, the level of hypothermia may affect age-related learning and memory performance as a significant factor. Moreover, hypothermia may also cause comorbidities including neurodegeneration. For example, in studies of disease models such as traumatic brain injury (TBI) accompanied by many comorbidities such as progressive neuronal damage, neurodegeneration, and inflammation (Golub & Reddy, 2022), test-induced hypothermia in addition to these comorbidities is considered an undesirable condition.

Unlike in previous studies, our study investigates the effects of ITI and water temperature values on memory performance, body weight, and body temperature in rats to determine the existence of possible species-specific variations in these variables. In addition, it is aimed to determine the optimal ITI that will enable accurate assessment of memory performance, improve animal welfare, and minimize potential side effects by examining the range of the waiting time values used in standard MWM protocols. Thus, our study will replicate and extend the data from previous studies by revealing the effect of different water temperature values at different intervals during the MWM protocol on experimental rats.

Materials and Methods

Subjects: In the study, 32 female rats of 6 months old Wistar Hannover strain (with an average weight of 220-260 g) were used. In our reference study, the greatest difference in body temperatures was observed between female mice (Iivonen et al., 2003). For this reason, female rats were chosen for the study. Water and food were provided as ad libitum. Animals were housed in room conditions with 50-60% humidity, 18-22 °C temperature, 15-17 cycles per hour ventilation, and a lighting cycle of 12 hours light and 12 hours dark. Groups of animals were formed according to the differences in waiting times between swimming trials (30-s and 13-min) and different temperatures of the water (20 ± 0.5°C and 24 ± 0.5°C). 32 rats were randomly divided into groups as follows:
Group: 30-s ITI / 20 °C WT (n=8)
Group: 30-s ITI /24 °C WT (n=8)
Group: 13-min ITI / 20 °C WT (n=8)
Group: 13-min ITI / 24 °C WT (n=8)

Experimental design. Animal experiments were carried out after approval (No:2022-04) from the Animal Experiments Ethics Committee of Istanbul Bagcilar Training and Education Hospital on 28.02.2022. The MWM test was applied to all animals to evaluate hippocampus-dependent memory performance. Before the protocol, the tails of all animals were painted and individually numbered. Each animal was weighed one day before starting the protocol. In addition, body temperatures were measured rectally twice a day, before and after the MWM test (as soon as 4 trials were completed for each animal). Basal body temperature is the first measurement of each day. The difference in body temperature (Δt) was calculated by subtracting the body temperature before the first trial from the body temperature after the last trial (Δt=initial measurement - last measurement). After the trials were done, animals were dried in cages with a heating source.

Morris water maze. The MWM was conducted in a standard water-filled pool with a diameter of 150 cm and a depth of 60 cm. It was filled with water to rise 1.5 cm above the 15 cm wide platform placed inside the pool. Every object in the room was kept in the same place from the beginning to the end of the experiment. The water temperature of the pool was fixed at 24 °C and 20 °C in accordance with the experimental groups, and the water has been blurred with black food dye. The pool was kept blurred throughout all learning trials and probe trials. In learning trials, rats were put into the water from the farthest point (South) to the quadrant (North) where the platform is located. Rats were allowed to learn the location of the platform by swimming for 60 seconds in a 150 cm diameter pool for four consecutive days and four times a day. The starting quadrants of the groups were counterbalanced between the 3 quadrants, except for the quadrant where the platform is located. Escape latency is defined as the time for the rats to find the platform. Escape latencies for all rats were recorded. After the learning trials were completed, to assess memory function, the platform was lifted and the duration the rat swam in the target quadrant, where the platform was located during the learning trials, was recorded

(day 5). Probe performance was defined as the time the rats spent in target quadrant after the platform was removed. All trials were recorded with a camera.

Statistical analysis: Statistical analyses were performed using SPSS software ver. 25.0 2 (water temperature effect: 20 °C and 24 °C × 2 (time between learning trials: 30-s-13-min) factorial design was applied. First, it was determined whether the data showed a normal distribution by considering the Shapiro-Wilk and histogram graphs. In the measurements in which the assumption of normality was met, two-way and mixed-design ANOVA tests were applied. In accordance with the results of Levene's test, Tukey or Games-Howell's post hoc test was used to identify the cause of the difference between the groups. The differences at the level of $p < 0.05$ were accepted as significant.

Results

Although the number of animals in the study was first determined as 32, 29 animals were included in the statistical analysis, as three had swimming problems. 2×2 ANOVA analysis was used to determine the effects of ITI and the water temperature on probe performance. The analysis yielded a statistically significant ITI main effect F (1, 25) = 7.191, $p = 0.013$, partial η2 = 0.223). The 13-min ITI group's (M = 21.5, SEM = 1.53) probe performance was better than 30-s ITI group's (M = 15.79, SEM = 1.48). The water temperature main effect and the interaction between ITI and the water temperature were not significant, $p > 0.05$.

Table 1. The effects of ITI and WT on MWM probe performance.

	MWM Probe Performance		
	n	x̄	SEM
Main effect of ITI			
30-s group	15	15.79[a]	1.48
13-min group	14	21.5[b]	1.53
Main effect of WT			
20˚C	15	19.65	1.48
24˚C	14	17.65	1.53
*ITI*WT interaction*			
30s-20˚C	8	16.88	2.02
30-s-24˚C	7	14.71	2.16
13-min-20˚C	7	22.43	2.16
13-min-24˚C	7	20.57	2.16
		p- value	
ITI		0.013	
WT		0.354	
ITI*WT interaction		0.944	

a, b = Values with different superscripts within each column are significantly different. ITI = Inter-trial interval; S.E.M = Standard Error of Mean; WT = water temperature. Probe performance was defined as the time spent in the target quadrant.

Table 2. Change in the body weight of the WT groups during the days

		Body Weight		
		n	x̄	SEM
WT	*Time*			
20˚C	1	15	246.8[a,d]	3.34
	2	15	243.8[b]	3.71
	3	15	244.7[c,b]	3.67
	4	15	245.9[c,a,d]	3.71
	5	14	247.3[d]	3.85
24˚C	1	12	237.9[a]	3.72
	2	12	236.2[a,b]	4.14
	3	12	234.5[b,c]	4.09
	4	12	233.0[c]	4.14
	5	12	233.8[c]	4.29
			p-value	
WT*Time interaction			<0.001***	

Note. *$p < 0.05$, **$p < 0.01$, $p < 0.001$, ***; a, b, c, d = Values with different superscripts within each column are significantly different. SEM = Standard Error of Mean; WT = Water temperature.

A mixed-design ANOVA was performed with body temperature, as the dependent variable, body temperature (day1-pre/post, day2-pre/post, day3-pre/post, day4-pre/post, and day5-pre/post) as the within-subjects, and ITI and the water temperatures as the between-subjects independent variables. As a result of the analysis, less decrease in the body temperature was observed in 13-min ITI group (M = 37.64, SEM = 0.11), compared to 30-s ITI group (M = 37,28 SEM = 0.11) (F (1, 25) = 5.114, $p = 0.033$, η2 =0.17). The interaction between time and the water temperature was significant (F (9, 225) = 2.842, $p = 0.043$, η2 = 0.102) (Table 3).

A 2×2 ANOVA was conducted to evaluate the effects of ITI and the water temperature on Δt. Results showed that 30-s ITI group (M = 1.13, SEM = 0.11) had a statistically significant decrease in Δt value compared to 13-min ITI group (M = 0.53 SEM = 0.12) (F (1, 25) = 13.528, $P = 0.001$, η2 = 0.351). Similarly, a statistically significant decrease in Δt value was observed in the 20 °C WT group (M = 1.06, SEM = 0.11) compared to 24 °C WT group (M = 0.59, SEM = 0.12) (F (1, 25) = 8.154, $P = 0.009$, η2 = 0.246). The interaction between ITI and the water temperature were not significant, $P > 0.05$.

Table 3. Change in the body temperature of the WT groups during the eight trials (day 1, day 2, day 3 and day 4) and probe phase (day 5)

		Body Temperature		
		n	x̄	SEM
WT	*Time*			
20˚C	1	15	38.4[a]	0.16
	2	15	35.92[b,f]	0.23
	3	15	37.87[c,d,e,g,i]	0.15
	4	15	37.51[d,c,e,g,i]	0.26
	5	15	37.9[e,c,d,g,l]	0.15
	6	15	36.38[f,b,h]	0.51
	7	15	37.67[g,c,d,e,j]	0.10
	8	15	37.29[h,d,f,j]	0.17
	9	15	38.01i[,c,d,e]	0.12
	10	15	37.43[j,d,g,h]	0.16
24˚C	1	14	37.87[a]	0.16
	2	14	35.93[b]	0.24
	3	14	37.97[c,a]	0.16
	4	14	37.01[d]	0.27
	5	14	37.96[e,a,c,h,j]	0.15
	6	14	37.56[f,a,c,d,e,g,h,i,j]	0.52
	7	14	37.63[g,a,c,d,e,f,g,i,j]	0.11
	8	14	37.61[h,a,cd,e,f,g,i,j]	0.18
	9	14	37.52[i,a,d,f,g,h]	0.12
	10	14	37.84[j,a,c,e,f,g,h]	0.17
			p-value	
WT * Time interaction			0.043	

a, b, c, d, e, f, g, h, i = Values with different superscripts within each column are significantly different. SEM = Standard Error of Mean; WT = water temperature. 1, 3, 5, 7, and 9th body temperature measurements were taken before the MWM trials, 2, 4, 6, 8, and 10th body temperature measurements were taken after the MWM trials.

Discussion

There have been many well-studied procedures for the optimal use of the MWM test or present new methods for behavioral analysis, such as providing the appropriate interval training and probe trials, and control procedures to evaluate non-spatial features (Vorhees & Williams, 2006) or the assessment of age-related cognitive deficit (Gallagher et al., 2015). However, the hypothermia-related issues of MWM have not been sufficiently discussed in the previous research. Our study revealed that this issue may play an important role in experimental studies. Therefore, we provide a protocol that minimizes hypothermia-related issues during the assessment of spatial memory. This study was designed specifically for the more effective implementation of serially applied MWM protocols. The results of our study show that differences in ITI affect memory performance. Particularly, longer ITI has been found to improve memory performance compared to shorter ITI. In addition, it has been observed that long-term exposure to differences in water temperature has an effect on body weight over time. Another finding is that a greater decrease in body temperature is observed in shorter ITI and 20 °C water temperature. The last finding is that the longer ITI compensates for the decrease in body temperature. As a result, the prolongation of the time between trials in young rats positively affects memory processes and body temperature remains more stable. In studies, the 30-s ITI in the MWM is preferred for both young and old rats for learning and memory assessment (Conn, 2011; Bizon et al., 2001). The results of our study reveal that a longer ITI should be preferred instead of the frequently used 30-60 second ITI in the assessment of the memory with MWM. However, only young animals were used in our study. It is thought that working in different age groups and different waiting times (5-10 minutes) will contribute to the literature.

The MWM test is a reliable test correlated with hippocampal synaptic plasticity (Vorhees & Williams, 2006). Studies have shown that intraoperative

Table 4. The effects of ITI and WT on Δt

	Δt		
	n	x̄	SEM
Main Effect of ITI			
30-s group	15	1.13[a]	0.11
13-min group	14	0.53[b]	0.12
Main effect of WT			
20˚C	15	1.06[a]	0.11
24˚C	14	0.59[b]	0.12
*ITI*WT interaction*			
30s-20˚C	8	1.47	0.47
30-s-24˚C	7	0.79	0.44
13-min-20˚C	7	0.66	0.47
13-min-24˚C	7	0.41	0.37
		P- value	
ITI		0.001	
WT		0.009	
ITI*WT interaction		0.2	

a, b = Values with different superscripts within each column are significantly different. ITI = Inter-trial interval; SEM = Standard Error of mean; WT = water temperature; Δt = Difference between body temperatures (initial body temperature measurement - last body temperature measurement).

hypothermia negatively affects spatial memory (Xu et al., 2022). The hippocampus is a brain region that is responsible for spatial memory functions (Bellmund et al., 2018) and is quite responsive to stress (Eichenbaum, 2000; O'Keefe & Nadel, 1979; Scoville & Milner, 1957). Synaptic plasticity, the ability of synapses to modulate their strength or efficacy of synaptic transmission, underlies learning, memory, and information processing in the brain (Mansvelder et al., 2019). A decrease in rat body temperature down to 28–30 °C is known to impair MWM spatial memory performance (Rauch et al., 1989). In audiogenic stress studies, MWM can be used to measure spatial memory, especially after the electrodes are placed in the hippocampus regions of the animal stereotactically (Kim et al., 2007). In stress-related studies mentioned, the addition of hypothermia to MWM may affect the stress-related reflections of learning abilities. Therefore, maintaining core temperature in a normal range is essential for avoiding MWM-related comorbidities.

Conclusion

Our study will provide valuable data for experiments using the MWM test. Especially in some disease model studies, increasing the resting time between swimming times will both reduce stress and be effective in reducing comorbidities. The minimum fluctuation in body temperature with increasing water temperature will provide an advantage for manipulations that may cause changes in body temperature, such as stress-related or intraoperative studies to be made in addition to behavioral experiments. In addition, the parameters studied in postoperative cognitive function studies that require minimal changes in body weights may benefit from these data in terms of the standardization of metabolic changes. Moreover, the standard protocol may be reorganized in a species-specific manner and ITI values determined with this perspective may be considered as a hypothermia-related comfort zone for animal welfare.

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