ORIGINAL ARTICLE

OPTIMIZATION OF MORRIS WATER MAZE PROTOCOLS: EFFECTS OF WATER TEMPERATURE AND HYPOTHERMIA ON SPATIAL LEARNING AND MEMORY IN AGED FEMALE RATS

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Abstract

Introduction: Aging is a highly complex process driven by a multitude of factors. The use of humans in aging research is complicated by many factors (ethical issues; environmental and social factors; long natural life span). Therefore, rats are common models for the study of aging and age-related diseases. The Morris water maze test is one of the most common cognitive tests in studies investigating age-related learning and memory. However, standardized protocols are lacking, which could influence results. This study investigated the effects of water temperature and inter-trial interval on physiological parameters, hippocampus-dependent learning, and memory in aged female rats performing the Morris Water Maze.

Materials and Methods: Thirty-two female Wistar Hannover rats, aged over 18 months, were divided into four groups based on water temperature (20 °C or 24 °C) and inter-trial interval (30 seconds or 13 minutes).

Results: Rats exposed to 24 °C demonstrated better spatial learning and memory retention than those at 20 °C. The interaction between inter-trial interval and water temperature significantly affected memory, with higher temperatures improving memory, especially with shorter intervals. Inter-trial interval alone did not significantly affect learning, but longer durations were associated with more stable body temperatures. Neither water temperature nor inter-trial interval significantly influenced body weight.

Conclusion: These findings emphasize the need to standardize environmental conditions in Morris water maze protocols to enhance research validity and reliability. Optimizing these protocols is crucial to upholding ethical standards and ensuring animal welfare, advancing more effective and scientifically sound practices in gerontological research, and fostering a better understanding of aging processes.

Keywords: Aging; Animal Welfare; Body Temperature; Memory and Learning Test; Spatial Memory; Morris Water Maze Test.

INTRODUCTION

Understanding the pathophysiology of diseases such as dementia, Alzheimer's, and sarcopenia that accompany aging, and developing treatment strategies for these conditions, is critically dependent on experimental models. Although a large number of experimental studies have been carried out in this area, their aetiology needs to be further clarified. However, in studies within the field of gerontology, the fragility of the aged animals used must be considered, and protocol details specific to geriatric animals should be standardized. Otherwise, inconsistencies in the clinical application of findings may arise due to the lack of standardization between experiments.

The Morris water maze (MWM) test is one of the most commonly used cognitive tests in research in the fields of age-related learning and memory loss (1,2). However, factors such as the level of care during test administration and the experience of the practitioner can influence the model animals' associated stress levels, potentially impacting test performance. Indeed, studies in both mice and rats have shown that heightened sensitivity to stress can negatively influence performance in the MWM test (3). Additionally, MWM protocols commonly result in varying levels of hypothermia due to prolonged exposure to cold water, which can further elevate stress levels. Uncontrolled hypothermia observed during MWM test performance may exacerbate stress effects, resulting in impaired cognitive performance (4,5). These discrepancies necessitate the development of standardized protocols to minimize confounding variables and improve the consistency and reliability of MWM test outcomes.

Increasing the standardization and reliability of animal experiments can also improve animal welfare. In the context of the MWM, minimizing the side effects of the tests requires a detailed examination of the associated hypothermia-related processes and subsequent protocol optimization according to specific criteria (e.g. gender, age, animal model, body weight, etc.). Gender differences in spatial cognition remain a controversial issue, as many studies have shown no differences (6), while some studies have reported that males generally outperform females in terms of some spatial abilities (7). Furthermore, the historical predominance of male animals in scientific research has resulted in the inadequate representation of female biological and behavioral responses, thereby hindering our comprehensive understanding of interspecies biological differences. Thus, the use of female rats in the present study aims to deepen the investigation into gender-specific responses, thereby expanding the scope of research findings.

Age is one of the critical factors that should be taken into account when designing behavioral tests or interpreting behavioral differences that can occur due to the effect of experimental applications (8). Several experimental studies have shown that aging increases anxiety levels in rats, and further negatively impacting cognitive abilities such as learning and memory functions (8,9). Indeed, studies have shown that older rats of both sexes must perform significantly more trials to learn the maze task than younger rats (6). Further, several studies have shown that older animals take longer to find the hidden platform, travel longer distances to find the platform, and may require more trials for learning (10,11). As a result of these longer learning times, older animals spend more time in the water before completion of the experimental period.

Studies have shown that extending the time between flotation tests prevents hypothermia by eliminating the net cooling effect of water, and further exerts positive effects on learning. Studies have further reported that this may change depending on age (4,5). As such, the level of hypothermia may influence age-related learning processes as a major factor, potentially introducing comorbidities with neurodegenerative effects. In particular, adding test-induced hypothermia to the already present

comorbidities, such as progressive neuronal damage, neurodegeneration, and inflammation seen in conditions like traumatic brain injury, further complicates the outcomes of MWM tests (12).

Given the above context, the present study aimed to examine the effects of different water temperatures (WT) and varying exposure intervals to these temperatures on hippocampus-dependent learning and memory processes in aged rats (over 18 months old). Unlike previous studies, the present study followed a protocol that involved placing the animals in heated drying cages between flotation sessions to minimize body temperature (BT) loss, thereby enhancing animal welfare throughout the procedure. This study also sought to explore the relationship between age—a key variable in assessing cognitive impairment—and learning, as prior research has identified a significant difference in learning associated with waiting times in adult rats (5). By examining this relationship, we aimed to standardize MWM waiting times for aged rats and to reassess these parameters from the perspective of animal welfare. This approach was designed to provide a more effective framework for MWM protocols, particularly those conducted in series. Moreover, this study emphasized the need for precise control over influencing variables such as waiting times and WT, which are often inconsistently reported or overlooked in prior research (13). Given that WT can vary considerably across studies (14), even minor differences in these parameters within what is considered a standard range, can significantly impact scientific outcomes, particularly in geriatric populations. This research underscores the necessity of carefully considering these variables to enhance the accuracy and reliability of results in aging studies.

When standardization between experiments is not achieved, and the characteristics of the population being modeled are neglected, inconsistencies in clinical applications of the findings may arise. The authors were particularly attentive to the fact that the same protocols used for both aged and young animals in MWM studies may not adequately reflect the unique needs of geriatric subjects (5,14). As a result, this study aimed to contribute to the field of gerontology by developing a detailed protocol specifically for aged animals, thereby improving the accuracy and reliability of aging research.

MATERIALS AND METHODS

Animals

This experimental study was approved by the Local Ethics Committee for Animal Experiments of Bağcılar Training and Research Hospital (Protocol no: 2023/13). After receiving ethical approval, the study was performed at the Experimental Research and Skill Development Center (BADABEM) of Bağcılar Training and Research Hospital.

Thirty-two female Wistar Hannover rats aged over 18 months, with an average weight of 400-450 g were used as the experimental animals. Rats were provided with water and feed ad libitum, under the following controlled conditions: humidity of 50-60% humidity, room temperature 18-22 °C, 15-17 cycles of ventilation per hour, and a 12 hour light/dark cycle. All animals were born and taken care of in the same laboratory and kept in the same housing conditions during the experiment. Animals were fed ad libitum and placed on a stainless-steel wire grid (PLEXX, Netherlands) with 750 ml drinker cups. Groups were housed in 425 x 265 x 180 mm polycarbonate conventional Type 3H cages (PLEXX, The Netherlands) in pairs.

In accordance with legislation and guidelines on the ethical use of animals, all subjects in this study were aged specimens housed in the laboratory that had previously been used for educational purposes. This approach was taken to minimize animal use, while ensuring compliance with animal welfare standards.

The 32 experimental rats were divided into four groups of 8 rats each, according to inter-trial interval (ITI) and water temperature (WT). The groups were

as follows: ITI of 30 seconds at WT 20°C, ITI of 30 seconds at WT 24°C, ITI of 13 minutes at WT 20°C, and ITI of 13 minutes at WT 24°C.

Experimental Design

The MWM test was applied to all animals to evaluate hippocampus-dependent learning and memory (1). To reduce stress and acclimate the animals, three days before the start of the tests, rats were kept in the experimental room for 15 minutes a day and moved to the room 1 hour before the tests. Each animal was weighed once a day before starting the protocol. In addition, BT was measured rectally twice a day, both before and after MWM (i.e., as soon as the 4 trials were completed for each animal). During rectal measurement, the experimentor ensured that there was no feces in the anus, and that the thermometer was placed 1 cm inside the anus.

Morris Water Maze (MWM)

The MWM protocol was performed in a 150 cm diameter, 60 cm deep standard water-filled pool. The 15 cm wide platform placed in the pool was filled with water so that the platform was submerged by 1.5 cm. All objects in the room were maintained in the same place from the beginning to the end of the experiment. The WT of the pool was fixed at either 24 °C or 20 °C, in accordance with the experimental groups. The water was opacified with black food coloring, and remained turbid during all exercises and tests. The MWM test comprised both learning and memory assessments. In the learning phase, rats were allowed to learn the location of the platform by swimming in a pool with a diameter of 150 cm for 60 seconds, 4 times a day over 4 consecutive days. The rats were released into the water from 3 of the quadrants, excluding the quadrant where the platform was located, according to the randomly determined release order. During each trial, the rat was placed in the pool, close to and facing the wall, and was never released from the same quadrant in the same day. The time it took the rats to find the platform was recorded, and these data were used to assess the learning function. To evaluate memory retention, the probe trial was conducted on the fifth day: in this trial, the platform was removed, and the time spent swimming in the target quadrant (where the platform was previously located) was recorded. After the protocol was completed, all animals were returned to BADABEM.

Statistical Analysis

The descriptive statistics were computed to summarize the central tendencies and dispersion. Normality was subsequently assessed using skewness, kurtosis, and histogram inspection to confirm compliance with parametric assumptions. Learning performance (LP) was evaluated over four consecutive days, with four trials performed each day. The mean scores for each day were calculated, resulting in phase scores labeled as Trials 1 through 4. A 4x2x2 Multivariate Analysis of Variance (MANOVA) for Mixed Measures was conducted, with LP as the dependent variable and WT (20 °C, 24 °C) and ITI (30 seconds, 13 minutes) as the independent variables. Memory performance (MP) was analyzed using a Two-Way Analysis of Variance (2x2 ANOVA), with memory scores as the dependent variable and ITI and WT as independent variables. Changes in basal BT (ΔT) were measured as the difference between the daily pre-trial and post-trial temperatures, while body weight (BW) was tracked to monitor physiological changes. These variables were finally analyzed with a MANOVA using a 5-level structure for daily measurements, with WT and ITI as independent variables. Post hoc analyses were performed using Bonferroni corrections. All analyses were conducted in SPSS (v26.0, IBM Corp.), with significance set at $p<.05$.

RESULTS

MANOVA for Mixed Measures was conducted to examine whether LP differed based on the ITI and WT levels. A statistically significant difference was observed between the means of LP at the different

WT levels (F[3, 84] = 3.102, p<.05, η^2 = .100). However, no statistically significant effect was found for ITI (F[3, 84] = 0.894, p>.05, η^2 = .031), or for the interaction between ITI and WT on LP (F[3, 84] = 2.333, p>.05, η^2 = .077) (Table 1 and Table 2).

ANOVA (2x2) was conducted to examine whether MP differed according to ITI and WT levels. The interaction effect of ITI and WT variables was found to significantly affect MP (F[1, 28] = 4.240, p<.05, η^2 = .132). Further, MP varied significantly across WT categories when not considering ITI (F(1, 28) = 5.956, p<.05, η^2 = .175); however, no significant variation in MP was observed when considering ITI alone without accounting for WT (F[1, 28] = 0.093, p>.05, η^2 = .003) (Table 3).

MANOVA for Mixed Measures was conducted to examine whether the changes in basal BT (ΔT) varied according to ITI and WT levels, yielding a statistically significant difference in the ΔT means across ITI levels (F[4, 112] = 6.909, p<.01, η^2 = .198). However, no significant differences were found across WT levels (F[4, 112] = 2.039, p>.05, η^2 = .068), nor was there a significant interaction effect on ΔT scores between ITI and WT levels (F[4, 112] = 2.068, p>.05, η^2 = .069) (Table 4 and Table 5).

MANOVA for Mixed Measures was conducted to examine whether BW varied according to the ITI and WT levels, with results indicating that neither the ITI levels (F(4, 112) = 1.501, p>.05, η^2 = .051) nor the WT levels (F[4, 112] = 1.834, p>.05, η^2 = .061) significantly affected BW. Furthermore, no significant interaction effect of BW was observed between ITI and WT levels (F[4, 112] = 0.224, p>.05, η^2 = .008).

Table 1. Descriptive statistics of MWM learning performance

	ITI Group	WT Group	n	M	SD
Trial 1	30 s	20 °C	8	53.41	6.79
		24°C	8	45.59	8.82
	13 min	20 °C	8	57.06	4.62
		24°C	8	42.91	13.79
Trial 2	30 s	20 °C	8	49.22	14.46
		24°C	8	43.66	11.39
	13 min	20 °C	8	48.94	18.19
		24°C	8	41.13	11.73
Trial 3	30 s	20 °C	8	34.34	17.28
		24°C	8	37.91	13.88
	13 min	20 °C	8	30.50	13.55
		24°C	8	38.94	15.81
Trial 4	30 s	20 °C	8	39.38	20.53
		24°C	8	30.06	8.96
	13 min	20 °C	8	17.62	7.78
		24°C	8	33.38	13.77

Note. ITI = Inter-trial Interval; M = Mean; SD = Standard Deviation; WT = Water Temperature.

Table 2. Effect of water temperature groups on MWM learning performance

		Learning Performance		
WT Group	**Time Point**	**n**	**M**	**SD**
20 °C	Trial 1	16	55.23a	5.92
	Trial 2	16	49.08	16.88
	Trial 3	16	34.42	15.13
	Trial 4	16	28.50	18.74
24°C	Trial 1	16	44.25a	11.27
	Trial 2	16	42.39	11.25
	Trial 3	16	38.41	15.81
	Trial 4	16	31.72	11.36
		df	**F**	**Partial η^2**
Main effect of WT		3, 84	3.102*	.100

Note. *p<.05; a = Values within the same column marked with the same superscript differ significantly; df = Degrees of Freedom; M = Mean; SD=Standard Deviation; WT=Water Temperature.

Table 3. Effects of water temperature and ITI on MWM memory performance

	Memory Performance		
	n	**M**	**SD**
WT Group			
20 °C	16	13.19	1.16
24 °C	16	17.19	1.16
ITI Group			
30 s	16	15.44	1.16
13 min	16	14.94	1.16
WT x ITI			
20 °C - 30 s	8	11.75a	3.50
20 °C - 13 min	8	14.63	3.66
24 °C - 30 s	8	19.13a	5.74
24 °C - 13 min	8	15.25	5.23
	df	**F**	**Partial η^2**
Main effect of WT	1, 28	5.956*	.175
Main effect of ITI	1, 28	.093	.003
WT X ITI	1, 28	4.240*	.132

Note. *p<.05; a = Values within the same column marked with the same superscript differ significantly; df = Degrees of Freedom; M = Mean; SD = Standard Deviation; WT = Water Temperature.

Table 4. Descriptive Statistics for Changes in Basal Temperature (ΔT) Between Trials

	WT Group	ITI Group	n	M	SD
Trial 1 ΔT	20 °C	30 s	8	2.55	1.17
		13 min	8	2.46	.98
	24°C	30 s	8	2.99	1.34
		13 min	8	1.48	.81
Trial 2 ΔT	20 °C	30 s	8	3.19	1.45
		13 min	8	1.25	1.01
	24°C	30 s	8	1.51	.98
		13 min	8	0.89	.98
Trial 3 ΔT	20 °C	30 s	8	2.28	1.30
		13 min	8	-.66	1.08
	24°C	30 s	8	1.44	.79
		13 min	8	.44	.68
Trial 4 ΔT	20 °C	30 s	8	1.48	1.73
		13 min	8	0.25	.38
	24°C	30 s	8	.98	.85
		13 min	8	-.01	.87
Trial 5 ΔT	20 °C	30 s	8	.21	.36
		13 min	8	1.19	.93
	24°C	30 s	8	-1.41	3.90
		13 min	8	-.11	.41

Note. ITI = Inter-trial Interval; M = Mean; SD = Standard Deviation; WT = Water Temperature; ΔT = Difference in body temperatures (initial body temperature measurement - final body temperature measurement).

Table 5. Effect of ITI on Changes in Basal Temperature (ΔT) Between Trials

			ΔT	
ITI Group	**Time Point**	**n**	**M**	**SD**
30 s	Trial 1 ΔT	16	2.77	1.23
	Trial 2 ΔT	16	2.35a	1.50
	Trial 3 ΔT	16	1.86b	1.12
	Trial 4 ΔT	16	1.23c	1.34
	Trial 5 ΔT	16	-.60	2.80
13 min	Trial 1 ΔT	16	1.97	1.01
	Trial 2 ΔT	16	1.07a	11.25
	Trial 3 ΔT	16	-.11b	1.04
	Trial 4 ΔT	16	.12c	.66
	Trial 5 ΔT	16	.54	.96
		df	**F**	**η^2**
Main effect of ITI		2.401, 67.234	6.909**	.198

Note. *p<.05, **p<.01, ***p<.001; a, b, c = Values with the same superscript within a column indicate statistically significant differences; ITI = Inter-trial Interval; M = Mean; SD = Standard Deviation; WT = Water Temperature, ΔT = Change in body temperature (initial body temperature measurement - final body temperature measurement).

DISCUSSION

The present study was conducted to examine the effect of changes in WT and ITI on hippocampus-related learning, memory processes, and physiological parameters such as basal BT and BW in aged female rats. Overall, our findings revealed that WT exerted a significant effect on LP; in particular, rats swimming at 24°C learned the location of the target quadrant more efficiently than those swimming at 20°C, particularly on the first trials. This result demonstrates the potential effects of conditions on neural plasticity and memory formation, and agree with the modulatory effects of stress on learning reported in previous studies (15,16). However, the ITI and the interaction between ITI and WT exerted no significant effect on LP. These results highlight the importance of considering environmental factors such as WT when optimizing MWM protocols. As these findings indicate that ITI duration alone is not a determinant of LP in aged rats, researchers should investigate alternative factors when manipulation of this variable does not have the expected effect. Further, it should be considered that this phenomenon may also be attributed to aging and cognitive impairments. Indeed, research on LP spans a broad age range, typically between 18 and 28 months. Additionally, it has been reported that the outcomes are closely associated with strain differences (17).

Rats swimming at 24°C showed superior MP to those swimming at 20°C, demonstrating the importance of the effect of the thermal environment on cognitive functions. This result is in line with the capacity of temperature to modulate neurobiological processes reported in previous studies (18). In particular, the positive effects of optimal temperatures on neuronal activity and synaptic plasticity have been frequently reported in previous studies (19,20). In contrast, we found that ITI alone did not significantly affect MP in aged rats. This is in contrast with a previous report in young rats, where MP improved as the ITI was extended (5). These findings indicate that the ITI alone may have a limited capacity to influence learning and memory in aged rats, possibly due to age-related frailty. Importantly, the interaction between ITI and WT significantly impacted MP. Specifically, higher WT improved MP, particularly when the ITI was short. This result highlights the complex interactions of environmental conditions and trial configuration on cognitive processes.

Another important finding was that ITI had a significant effect (η^2 = .198) on the basal BT of aged female rats. In particular, longer ITI durations decreased the changes in BT, indicating that ITI plays a prominent role in temperature regulation and physiological adaptation processes, which is similar to the findings in studies with mice (4). However, WT and the interaction of WT and ITI had no significant effect on BT changes, suggesting the possibility that WT did not have the expected effect on the animals' capacity to regulate BT, that the temperature ranges used were not sufficiently different, or that both groups were old and had similarly low thermoregulatory capacity (21). For this reason, it is important for further studies to replicate this study, but including different age groups.

ITI did not show a significant effect on LP and MP, suggesting that the effects of this variable on cognitive performance may be limited. Conversely, the fact that ITI had a significant effect on changes in basal BT indicates that this variable may affect the thermal comfort of animals, and thus could have important effects on animal welfare.

Finally, we found that ITI and WT are not significant influencing variables of BW in aged rats. BW is considered an important indicator of general health and well-being. In animal experiments, while changes in BW can be triggered by factors such as stress, disease, or nutritional deficiency (22). In the present study, modulation of the ITI and WT did not significantly impact BW, indicating that these factors do not have the potential to induce stress or other negative physiological effects in aged female rats.

However, this outcome may also reflect the positive effects of our ensurance of animal welfare, such as providing warmth and facilitating adaptation throughout the experiment.

The results of this study have significant implications for the optimization of the protocols used in the MWM test. As the present study shows, the standardization of environmental variables such as WT can have a significant impact on the consistency and reliability of experimental results. The different WTs used in the MWM test could introduce variations in research results; as such, the development of standardized protocols is important to ensure the comparability and reproducibility of scientific findings.

The aged female rats used in this study are important as a model sensitive to cognitive changes associated with aging. Indeed, older rats may show more sensitive responses than younger individuals, which requires the application of specialized protocols, particularly when investigating this population (23).

Scientific research has historically focused on male animals, a trend which has resulted in a poor understanding of the biological differences between the sexes (24). Therefore, the present study aimed to improve the biological validity of the MWM test using female rats, which are generally considered to be at a disadvantage in terms of factors such as thermoregulation. Furthermore, by using older female rats, this research supports the effort to provide a more inclusive and balanced body of knowledge in the scientific community. Tailoring MWM protocols specifically for this population could maximize the scientific accuracy of such studies by increasing the reliability and reproducibility of experimental results. Further, this study emphasizes the importance of rigorously managing environmental factors in cognitive and physiological experiments on aged rats to improve scientific accuracy and animal welfare. This research highlights that experimental designs should be optimized to take into account the effects of variables such as ITI and WT on animals, thereby providing researchers with ways to improve the thermal comfort and overall health of animals. This approach could contribute to the adoption of more ethical and humane scientific methodologies, particularly when applied to animals impacted by sensitive processes such as aging.

Moreover, the results of this study underscore the critical need to refine experimental protocols for aged animals in gerontological research, given their increased vulnerability compared to younger subjects. Rather than applying standard protocols designed for younger animals, researchers should tailor methodologies to the specific needs of aged models. This approach aligns with recent human studies, which point to molecular damage as a key factor in age-related phenotypes (25). Furthermore, the shorter lifespan of laboratory animals allows for accelerated investigation into aging, offering valuable insights into mitigating molecular damage and developing interventions that can slow aging processes (26). Ensuring the standardization of aging, as a physiological process, in experimental studies will significantly contribute to research in the field of gerontology.

While this study offers a comprehensive methodological approach, it is not without limitations. Most importantly, the difficulty in sourcing aged animals constrained the sample size, thus limiting the generalizability of the findings. Additionally, due to limited laboratory facilities, we were unable to perform detailed measurements of swimming strategies and speeds using automated systems. Instead, manual measurements were conducted using a camera, which may have introduced some variability in the data collection process. Previous studies have further demonstrated that animals may adopt a variety of swimming strategies during tests, including search strategies such as thigmotaxis, random search, scanning, chaining, focal search, focal wrong, perseverance, and direct search, as

well as non-search strategies such as circling and floating (27). The inability of the present study to capture these nuanced behaviors may have affected the depth of our behavioral analysis as well as the interpretation of MP. Future research could expand on our results by incorporating different gender and age groups, thereby contributing to the development of standardized protocols. Nevertheless, despite these challenges, the findings of the present study underscore the critical importance of rigorously managing environmental factors and optimizing experimental designs when performing experiments with aged populations. This approach not only enhances the reliability and reproducibility of scientific outcomes, but also aligns with the ethical imperative to ensure the well-being of animal subjects. By advancing standardization of the experimental investigation of aging, this research contributes to the broader field of gerontology, offering insights that may facilitate the development of more effective and humane methodologies to study the physiological processes of aging.

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