Late-onset calorie restriction worsens cognitive performances and increases frailty level in female Wistar rats

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Abstract

The current study aims to determine the potential benefits of calorie restriction (CR), one of the most promising paradigms for lifespan and healthspan extension, on cognitive performances in female Wistar rats during aging. As a measure of a healthspan, we evaluated the effects of different onset and duration of CR on frailty level.

Female Wistar rats were exposed to either ad libitum (AL) or CR (60% of AL daily intake) food intake during aging. Two different CR protocols were used, life-long CR with an early-onset (EOCR) that started at the adult stage (6 months) and 3-month-long CR, started at the middle (15 months) and late-middle (21 months) age, thus defined as a late-onset calorie restriction (LOCR).

The effects of CR were evaluated using open field, Y-maze, and novel object recognition tests. We broadened two tools for frailty assessment currently in use for experimental animals, and in alignment with our previous study we created a physical-cognitive frailty tool that combines both physical and cognitive performances. Our results clearly showed that CR effects are highly dependent on CR duration and onset. While a life-long restriction with an early-onset has been proven as protective and beneficial, short-term restriction introduced at late age significantly worsens an animal's behavior and frailty. These results complement our previous study conducted in males and contribute to the understanding of sex differences in a response to CR during aging.

Keywords: aging, memory, sex, nutrition, healthspan

Introduction

Humans have long sought the elixir for long life. However, the downside of a prolonged lifespan is the greater likelihood of disease and disability. Therefore, the main aim of every anti-aging intervention should be an extension of the healthspan, a disease-free period of life that could be quantified by measuring frailty level. Often conceptualized as a state of late-life decline, frailty is defined by the loss of physiologic reserves resulting in the extreme vulnerability of the individual (1). Along with physical decline, cognitive impairment most likely acts synergistically to promote the progression of disability (2,3). The concept of 'cognitive frailty' has been proposed almost a decade ago (4) and is defined as a simultaneous presence of mild cognitive impairment (MCI) and physical frailty (evaluated based on the Fried phenotypic model (5)). A number of human studies have shown an interplay between physical frailty and cognitive impairment, pointing out that physical frailty could predict the prevalence of cognitive impairment, while frail people were prone to higher cognitive vulnerability (6). In an attempt to carry out timely interventions, cognitive frailty could enable the early identification of individuals predisposed to cognitive deterioration and physical frailty.

To grasp the nature of frailty, tools for frailty assessment in humans, such as Rockwood's frailty index (FI) (7) and Fried's frailty phenotype have been translated into rodents (8-14). The main purpose of this translation was to enable testing of various anti-aging interventions, as frailty is not an inevitable consequence of aging, but rather a reversible geriatric syndrome (15). As such, it could be influenced by various anti-aging

interventions including calorie restriction (CR), whose efficacy in preventing an increase in frailty score (FS) in aging Wistar males we evaluated previously (16).

Since its inception, CR has become known as the "fountain of youth" extending the lifespan and healthspan across taxa (17). Although CR stands as a non-pharmacological intervention with the ability to deliver benefit at various levels, recently focus was slightly shifted towards the shortcomings that CR could bear. Emerging evidence points to the several determining factors for CR effects, such as the age, health status of the individual, the length, and the level of the food restriction (16,18). Controversy still surrounds the age at which CR achieves its most prominent effects since an increasing number of data implies that the use of CR in very young and old age may reduce the length and quality of life (16,19). In addition, special attention has been recently paid to sex differences in various age-related processes, including the identification of frailty biomarkers (20) and the efficacy of various anti-aging interventions (21). Namely, it has been shown by Kane et al. (21) that CR was ineffective in changing frailty index during aging in female mice of two different strains, while it was effective in males. Such an important difference in the efficacy of CR as an anti-aging intervention prompted us to extend our previous research in male Wistar rats (16), by evaluating changes of frailty level in response to different onset and duration of CR in aging female Wistar rats. As linking frailty status with cognition while assessing risk factors for dementia in older adults is lately in the spotlight (22), we expanded the unique physical-cognitive frailty score we previously created in animals (16). We detected that the outcome of CR was dependent on the age of the subject at the outset of diet and its duration. Interestingly, when compared to our previous data on male rats (16), we noticed a certain level of disparity between sexes in response to food restriction.

Methods

Animals and Treatments

Female Wistar rats were used in this study (n=50). Animals were bred at the Institute for Biological Research "Sinisa Stankovic" Vivarium, an animal SPF level 1 facility (https://www.rarc.wisc.edu/tools_and_guides/facility_designation_for_rodents.html). Animals were housed in groups of 2-3 in standard polycarbonate cages with wire bar cage lids and were maintained in a 12-h light/dark cycle (lights from 06:00h to 18:00h) at a standard room temperature and humidity, with *Ad libitum* (AL) access to food during the first six months of life. Tap water was available AL constantly. All animal procedures were approved by The National Ethic Research Committee (#323-07-03065/2020-05).

Rats were subsequently separated into two groups based on the body weight balanced random allocation scheme: AL animals, with free access to the food during the entire experiment, and calorie restricted (CR) groups (Figure 1). Calorie restricted animals were exposed to a limited daily feeding experimental paradigm (23), in which rats were receiving a daily allotment of food that represented a fixed percentage (60%) of the mean AL daily intake of a standard laboratory chow (manufacturer VZS Stocna hrana d.o.o, Subotica, Serbia). Such a limited dietary regimen was introduced either at 6 months of age and lasted until the animals were 18-, or 24-month-old (early-onset CR, EOCR) or CR started at 15 and 21 months of age and lasted for 3 months (late-onset CR, LOCR). Animals have been acclimated gradually to this restriction paradigm. First, daily food consumption for the groups predicted to undergo CR was measured for a week before introducing CR and the average daily consumption was calculated; the data are given in the online-only supplementary eTable 1. Further, this daily average amount of food was reduced by 10% every two days, so after 7 days their food consumption decreased by 40%. Animals were fed at 3 pm during the light

cycle. Body weight was monitored regularly and the body weight curves are given in the online-only supplementary eFigure 1. The general wellbeing of animals was monitored daily.

AL animals were used as controls for evaluating the effects of CR, while the 6-month-old AL group served as a control group solely for the effects of aging. Behavioral testing was performed in all experimental groups at 6 (adult), 18 (middle-aged), and 24 (aged) months of age. The number of animals per group varied from 6 to 9.

Y maze test

The Y-maze test has been performed as it was previously described (16). Briefly, animals were allowed to freely explore the two arms of the Y-maze during a 10-minute session (first trial), without any reinforcements, while the third arm (novel arm) was blocked off. After an intertrial interval of 1 hour, rats were allowed to freely explore all three arms of the maze for the next 10 minutes (second trial). A total number of entries, entries into the 3rd arm (four paws had to be inside the arm for a valid entry), and time spent in a third arm was monitored with a video camera, and spontaneous alternations, % of 3rd arm entries and percentage of successful alternations at levels significantly above chance (50%) were calculated based on the formulas as previously described (16,24). Rats that made fewer than 9 total arm entries during 10 minutes of observation were excluded from the analysis.

Novel object recognition task

A Novel object discrimination test (NOR) was used for short-term and long-term memory assessment, and methodology was modified from Mathiasen and DiCamillo (25). In brief, rats were habituated to an empty test arena of the open-field (OF) (44.2x43,2x20cm) for 30 min during 3 consecutive days before NOR testing. Experimental procedure included four sessions: I) Pre-trial habituation: animals were left to freely explore the empty OF arena

for 10 min on the testing day II) familiarization trial: two identical objects (A+A) were placed in opposite corners of the arena to be explored by animals for 10 min; III) Choice trial 1: After 1h of intertrial interval animals were exposed to short-term memory testing for 10 min, where one object from familiarization trial was replaced by a novel one while the other was left untouched (A+B); IV) Choice trial 2: After 24h intertrial interval animals were exposed to long-term memory testing for 10 min, where one object from familiarization trial was replaced by a novel one while the other was left untouched (A+C).

Animals were monitored with a video camera mounted above the experimental arena, connected to a computer to record a video file, and time spent exploring objects was measured with two timers. Exploration ratio and discrimination index were calculated regarding time spent exploring new versus familiar object, using the following formulas: exploration ratio=t novel/(t novel+t familiar); discrimination index=(t novel-t familiar)/(t novel+t familiar) (26). Animals that did not fulfill the baseline level of investigation set at minimum of 20 seconds of exploration for both objects during 10 min of testing were excluded from analysis, as it cannot be confirmed they spent enough time exploring to learn/discriminate.

Open field test

The activity of rats was recorded in Opto-Varimex cages and analyzed using AutoTrack software (Columbus Instruments, OH) as previously described (16). Briefly, rats were allowed to freely explore the open field test arena for 10 minutes and total distance traveled (DT), total duration of movement-ambulatory time (AT), percent of total time spent in moving (T/AT), the average velocity of movement (DT/AT) and rearing frequency, i.e., vertical activity (V1B) were measured.

Frailty Score

We calculated the frailty score based on the "Valencia score" developed by Gomez-Cabrera and co-workers (13) that was further adapted to create a unique physical-cognitive FS, by combining the measurements of both physical strength and cognitive status. Five parameters from the open field test were used in a combination with two NOR parameters and five Y-maze parameters (eTable 2). To compare the FS among experimental animals the "frailty groups" were formed as follows: for the effect of aging, we calculated how frail are 18- and 24-month-old AL animals in comparison to 6-month-old AL rats; for the effect of different feeding regimens, we paired the particular CR group with the appropriate AL group: 18 m AL with 18 m EOCR and 18 m LOCR, 24 m AL with 24 m EOCR and 24 m LOCR. Following the "Valencia score" method (13) for calculating FS, we established 20% as a cut-off point for all parameters used (16). Animals with behavioral performances in the lowest 20% were considered frail. The calculated values for 20% cut-off for each parameter during aging and under various feeding paradigms are given in eTables 3A and 3B, respectively. The FS for each "frailty group" was calculated as follows: a total number of tests failed by the animals at each experimental group (A) divided by the total number of tests performed by the same group of animals (B) and expressed as a percentage (13).

Clinical Frailty index

Calculation of a frailty index (FI) was based on the previous work (10,11,20). We included 23 parameters in our scale, 21 regarding the condition of different organ systems, and discrimination index and spontaneous alternations as 22nd and 23rd parameters, respectively (eTable 4). Each animal was assessed for a brief clinical examination and the severity of each deficit was rated with a simple scale: 0 (no sign of a deficit), 0.5 (mild deficit), and 1 (severe deficit) (eTables 5A and 5B). Having in mind that among studies, the

lower point of discrimination index value indicating novelty preference, varies usually from 0.2-0.5., we created the following scale for FI: discrimination index value of 0.5-1 was scored as 0, the value of 0.2-0.5 was scored 0.5 and value ≤0.2 was scored as 1 (27) (eTable 4). For spontaneous alternations, values higher than 50% chance were scored as 0, values that were lower than 50% were scored 0.5, and animals that had less than 9 entries during an experiment were scored 1 (eTable 4). All these values were then summed, and the total was divided by the number of parameters measured to provide a frailty index score between 0 and 1 for each animal.

Considering that weight loss is the expected outcome of CR, we excluded the body weight for the FS and FI calculation.

Statistical analysis

For Y-maze and NOR test results, statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple-comparison post hoc test, or nonparametric Kruskal-Wallis test followed by Dunn's post hoc test for the effects of aging and different feeding regimens. For evaluation of alternation above chance, one sample t-test was used. Analysis of FS and FI was performed by Pearson's chi-squared test, whereas data for each of the parameters was performed using G-test with Williams correction for a small number of samples. Statistical analysis and figures were created using GraphPad Prism version 7 (San Diego, CA). Values are shown as mean ± SEM and considered significant if $p<0.05$.

Results

The effect of different CR onset and duration on spatial memory during aging

To investigate spatial memory in rats during aging and under the influence of CR, we employed the Y-maze test. Statistical analysis of Y-maze parameters revealed significant changes during aging and upon CR regimens (Figure 2). One-way ANOVA confirmed the significant effect of age on spontaneous alternations value [$F_{(2,14)}$=5.327, p=.02] and the number of 3rd arm entries [$F_{(2,14)}$=5.263, p=.02] but not on time spent in the 3rd arm (Figure 2, white bars). Dunnett's multiple comparisons test showed a significant decrease in spontaneous alternations in the 18- and 24-month-old AL group (p=.02, p=.04, respectively) and in the number of 3rd arm entries of the 24-month-old AL group (p=.04) in comparison to the young, 6-month-old rats.

The value of spontaneous alternations in 18-month-old EOCR animals was significantly increased by 46% (*post hoc* p=.02) in comparison to the age-matched AL control group [$F_{(2,13)}$=4.8, p=.03] (Figure 2A, light-gray bars). The positive influence of EOCR was also evident in the 24-month-old group, where the increased number of entries and time spent in the 3rd arm [$F_{(2,14)}$=10.37, p=.002, p=.0003, respectively] was detected (Figure 2B and 2C, right panel, light-gray bars). The number of entries in the 3rd arm was increased by 72% (*post hoc*: p=.006) in comparison to controls. On contrary, the LOCR regimen had a deleterious effect on Y-maze performances, decreasing the time spent in the 3rd arm in 24-month-old animals by threefold [p=.0003] in comparison to the age-matched AL group (*post hoc* test, p=.04) (Figure 2C, right panel, dark-gray bar).

One sample t-test showed that 6AL, 18EOCR, and 24EOCR groups had a percentage of successful alternations at levels that were significantly above chance (50%) (6AL:p=.02,

18EOCR:p=.02, 24EOCR:p=.03) (Figure 2A), indicating preserved spatial memory is in these groups.

The effect of different CR onset and duration on short-term and long-term memory during aging

Next, we determined the effect of age and CR duration on non-spatial memory performance in the NOR test. Familiarization trials did not show side or object preference within groups (eFigure 2). Using the exploration ratio formula, we observed changes in short-term memory performance under the influence of age [$F_{(2,16)}$=12.20, p=.0006]. Dunnett's multiple comparisons test showed that 18- and 24-month-old AL animals had a lower exploration ratio in comparison to 6-month-old AL animals (p=.0003; p=.01, respectively) (Figure 3A, white bars). Similar results were observed during the long-term memory assessment. Aging led to an impaired recognition performance [$F_{(2,15)}$=4.549, p=.03] as Dunnett's multiple comparisons test showed a lower exploration ratio of 18- and 24-month-old AL animals (p=.03; p=.05, respectively) (Figure 3B, white bars).

Both EOCR and LOCR regimens had a protective effect on STM performance [$F_{(2,15)}$=5.565, p=.02], and both increased exploration ratio values in 18-month-old animals (*post hoc* test p=.01; p=.05, respectively) (Figure 3A, central panel). However, in the 24-month-old groups, no change in exploration ratio value was noticed after exposure to EOCR, while the deleterious effect of LOCR was observed [$F_{(2,15)}$=4.868, p=.02]. Namely, the lower memory performance was detected in the 24-month-old LOCR group in comparison with age matching control (*post hoc*: p=.03) (Figure 3A, right panel, dark-gray bar).

Preserved long-term memory performance was observed in 18-month-old females as a result of the EOCR regimen [$F_{(2,12)}$=6.668, p=.02] as a significantly higher exploration ratio in comparison to the 18-month-old AL females (p=.05) has been detected in this group

(Figure 3B, central panel, light-gray bar). Similarly to the negative effect of LOCR that was observed on the short-term memory, a decreased exploration ratio in the 24-month-old LOCR group [$F_{(2,14)}$=3.73, p=.04] was also detected in the long-term memory trial (*post hoc*: p=.04) (Figure 3B, right panel, dark-gray bar).

The effect of different CR onset and duration on FS during aging

Parameters from OF, Y maze, and NOR tests were combined to determine the physical-cognitive frailty level of female rats. The influence of aging itself and different dietary paradigms on each of the 12 parameters was analyzed separately and later used to calculate FS (see Methods section). Increased frailty score was detected in 18- and 24-month-old animals, due to a negative impact of age. In these two groups, an increased percentage of animals that scored in the lowest 20% for several parameters was detected (DT, AT, number of 3rd arm entries, and the total number of entries, eFigures 3 and 4), but no change was observed in the exploration ratio (% of success) (eFigure 5). FS increased about threefold in 18- and twofold in 24-month-old animals in comparison with 6-month-old animals (6AL: FS=12, 18AL: FS=30.8, 24AL: FS=26) (*post hoc*: p=.009; p=.008, respectively) (Figure 4A).

In 18-month-old animals, both EOCR and LOCR had beneficial effects on frailty level (18AL: FS=75, 18EOCR: FS=6.6, 18LOCR: FS=17.7) and significantly decreased FS in female rats (p<.0001; p<.0001, respectively). A protective effect was especially evident in the EOCR group (Figure 4C, light-gray bar). Both EOCR and LOCR increased DT, V1B, and AT values and percent of time spent moving (eFigure 6). Additionally, EOCR increased spontaneous alternations and long-term memory exploration ratio values (eFigures 7 and 8), while LOCR increased velocity, but decreased the percentage of 3rd arm entries (eFigures 6 and 7). Interestingly, the beneficial effect of the lifelong EOCR regimen seemed to fade away

by the age of 24 months, as no effect on examined FS parameters was observed in this group (eFigures 9, 10, and 11). On the other hand, the LOCR regimen in 24-month-old animals lowered the number of 3rd arm entries and time spent in 3rd arm, (eFigure 10), resulting in significantly increased frailty score for about threefold (24 AL: FS=18.2, 24EOCR: FS=15.9, 24LOCR: FS=51.9) (*post hoc*: p=.002) (Figure 4D, dark-gray bar).

The effect of different CR onset and duration on clinical FI during aging

Clinical FI was calculated using 23 parameters scale (eTable 4). A negative impact of age was proven once again [p<.0001], with FI value for 18-month-old and 24-month-old animals being higher for five- and fourfold (respectively) in comparison to 6-month-old animals (6AL: FI=0.04, 18AL: FI=0.2, 24AL: FI=0.15). Namely, both 18-month-old and 24-month-old AL groups had a higher percentage of frail animals than 6-month-old ones (*post hoc*: p=.002, p=.006, respectively) (Figure 4B). Eighteen months was the age point where positive outcomes of both EOCR and LOCR could be observed [$F_{(2,13)}$=5.107; p=.02] (18AL: FI=0.2. 18EOCR: FI=0.04, 18LOCR: FI=0.06). At this age point, the *post hoc* test showed significantly decreased FI in EOCR and LOCR groups (p=.02, p=.03, respectively) (Figure 4D). At the age of 24 months however, only EOCR had effect on FI [p=.05] (24AL: FI=0.15, 24EOCR: FI=0.07, 24LOCR: FI=0.18). Namely, the EOCR regimen was able to maintain lower FI even in 24-month-old animals (*post hoc*: p=.05), while LOCR had no effect in this age group (Figure 4F).

Among examined frailty parameters those that were most under the deleterious influence of age were incidence of tumors, hearing loss, coat condition, discrimination index, and spontaneous alternations (eTable 5A). At 18 months EOCR succeeded to prevent impairments in all examined parameters, apart from coat condition and discrimination index (eTable 5B). LOCR was also very protective and failed to preserve impairments only in coat

condition, body condition score, and discrimination index (eTable 5B). At 24 months, EOCR still had a strong protective effect. However, impairments in this group were still common for coat condition, body condition score, head tilt, exophthalmos, and discrimination index (eTable 5B). As for the negative effect of LOCR, the most influenced parameters were hearing loss, discrimination index, and spontaneous alternations (eTable 5B).

Discussion

Although it is widely accepted that CR is effective in preventing age-related changes, there is a persisting doubt whether the CR can mitigate cognitive decline as one of the main features of the aging brain. Herein, we extended a study in male Wistar rats previously conducted (16), addressing now how different onset and duration of CR influence cognitive performances of female Wistar rats during aging. As both cognitive decline and increased frailty are closely related to the aging process, we expanded the unique frailty index and frailty score by including additional cognitive components.

In recent years, numerous studies pointed to sex differences in various age-related processes. Herein we have shown that frailty level increases significantly in female Wistar rats during aging and that it can be modified by CR. However, comparing current results with the study in male Wistar rats (16) we detected that dynamics of age- and CR-induced response were somewhat different in females. While notable age-related changes of Y-maze parameters were detected solely at 18 months of age in males, in females we detected age-induced alternations mainly at 24 months of age. This indicates that female Wistar rats are probably more resilient to the age-induced decline in spatial memory than males. Both males and females were under a significant beneficial influence of long-term EOCR, which was evident from the higher inquisitive behavior detected in Y-maze. This effect was also noticed in elevated spontaneous alternation score that was significantly above 50% chance only in 6-

month-old controls and EOCR group of females, indicating that only long-term early-onset CR had a potential of improving spatial memory during aging. Interestingly, a short-term CR with late-onset had an undesirable impact on cognitive performances *per se* in females, which was not the case in males (16). Namely, LOCR reduced the time spent exploring a novel arm of Y-maze, which was not affected by the age itself. This reveals completely new insight into the potential of CR to modify the aging process, indicating the possibility that CR affects different cognitive abilities/forms of memory in different ways and a sex-dependent manner.

Cognitive performances were further evaluated with the novel object recognition task, considered as a very sensitive test for detection of subtle changes in declarative memory of animals, and is shown to be strain-, age- and gender-dependent in rodents (28). This test relies on an innate preference of animals for novelty where discrimination between the novel and familiar object involves hippocampal and cortical activity. Similar to age-related impairment in short-term memory observed in the Y-maze test, an age-related decrease in both short-term and long-term memory assessments of the NOR test was also detected, which is in accordance with previous studies on Fisher-344 rats (29). However, in comparison to the beneficial effect of EOCR noticed mainly at 24 months of age in Y-maze, in the current study we detected a significant beneficial impact of EOCR in the NOR test earlier, at the age of 18 months. These results imply that EOCR differently affects spatial and declarative memory during aging. At 18 months of age, EOCR seems to be protective regarding both short- and long-term memory in NOR assessment. LOCR also had different influences on non-spatial and spatial memory. While it had no effect in the 18-month-old group in the Y maze test, a protective effect of LOCR in the NOR test was scored, although only for short-term memory. Previous studies have shown that different molecular mechanisms underlie short- and long-term memory (30). Likely, 3-month-long LOCR implemented in middle-aged animals is not capable of reversing age-induced changes in long-term memory.

In contrast to 18-month-old animals, at the age of 24 months, both declarative (short- and long-term memory) and spatial memory has undergone a deleterious effect of LOCR, implying that old age is a life period when introducing CR could trigger a significant cognitive decline.

Effects of various feeding regimens on cognitive abilities presented above were further evaluated with frailty assessment. To include as many as possible body deficits associated with aging into frailty measurements, we united common parameters used for Fried's or Rockwood's frailty assessment with cognitive parameters from Y-maze and NOR testing. In assessing frailty, we have used both approaches (Fried's frailty score and Rockwood's frailty index) commonly used for frailty assessment, as we previously demonstrated in transgenic mice (5xFAD model) that these two tools do not necessarily detect the same animals as frail (31). Such outcome was not completely unexpected, considering that FS and FI are reflecting a decline in various body systems and functions, but this finding stressed the exact need for a more uniform evaluation of frailty level. We consider that adding the cognitive component as an additional parameter could significantly add to the reliability of frailty evaluation and would lead to more uniform results between studies that use different frailty assessments. This is especially true when it is needed to evaluate the effects of interventions that change cognitive performances, like CR herein.

In alignment with the conclusion obtained in the study with males (16), aging significantly increased frailty levels in female Wistar rats, although without a typical pattern. Namely, we haven't detected a difference between the frailty levels of 18- and 24-month-old females. This observation has been confirmed by both frailty tools. Almost the total lack of literature data about age-related frailty in female rodents, unfortunately, disables comparison of these results. There are no studies in female rats, while previous studies on aging female ICR/CD1 (32) and C57BL/6 mice (33,34), although showing certain age-related differences,

was conceptualized in the manner that comparing frailty levels between different aging groups was impossible, in addition to the different time points those authors investigated in comparison to us. Similar is the case with the study of Kane et al., (21), where solely the sex differences in a response to anti-aging interventions were assessed. We can only speculate that females reach their maximal frailty level earlier than males, which can be an additional component of the male-female health survival paradox, according to which females live longer despite higher frailty level (35).

Long-term EOCR had a recognizable protective effect as it fully prevented an age-related increase in frailty, while a short-term LOCR introduced later in life had contradictory effects. Although protective when it was introduced at 15 months of age, LOCR that started at 21 months led to a 3-times higher FS in comparison to AL age-matched rats. This clearly shows that for a beneficial effect, a restricted feeding regimen has to be introduced until a certain time point in life, likely by the late-middle-age period; otherwise, it will furthermore stress an organism with already impaired homeostasis due to the aging process itself.

The pivotal role that the age of the subject plays in the CR outcome was evident from the studies demonstrating CR effects on life extension in rodents (18). Calorie reduction initiated in 18-month-old rats completely failed to increase the median lifespan (36), while late initiation (24-month-old animals) in the three commonly used experimental mouse strains (C57BL/6, DBA/2, and B6D2F1), even significantly increased the mortality rate (37). A recent review also highlighted evidence that CR effects vary according to intensity, duration, and the period of CR, showing benefits on cognition in various experimental models, starting from rodents, to flies, and baboons, only when started early in life (38). Furthermore, even the short-term food restriction was proved to be effective in the preservation of learning and memory processes in the cases of age-related (Alzheimer's and Parkinson's diseases) pathologies, if applied in young adult PDAPP-J20 mice and mice with

Tau pathology (39,40). Our study shows that 3-month-long CR could have an opposite outcome when started at various periods of life. The chosen time points, 15 and 21 months of age have been considered as separate phases in rodent life; thus, in the first case CR can be characterized as an adult-onset CR, while the latter is old-onset CR (23). As previously observed in our research, this period (18 - 21 months of age) in rodents' life has been shown as a very sensitive one, when the deleterious effects of age start to appear. The most prominent changes include a decline in the nervous system structure and function, starting from the expression of synaptic proteins to the disruption of brain cholesterol homeostasis (41-44). In the study by Vaughan et al. (23) the authors suggested that there may be a "level of maturity, or a stage during the aging process, after which caloric restriction no longer increases longevity." Similar could be assumed for the effects of CR on cognition; besides a critical role that CR intensity, onset, and duration have, the main determinants of the CR outcome are physiological characteristics and health status of the individual, on which age has a significant effect (18,45-47).

Sex also influences the rate of aging and the responses to many anti-aging interventions including CR (21,48). For example, while CR that lasted from 6 months of age to 19 months of age significantly decreased age-related FI in male C57BL/6JJ and DBA/2J mice, it was ineffective in modulating frailty in females of the same age (21). We noticed similar disparity but later in life. While in male Wistar rats adult-onset CR was effective in decreasing FS at 24 months of age (16), it was not the case in females. Very recent work from Quirós Cognuck et al. (49) could add some missing links between the age, sex, and the effect of CR. Namely, this study demonstrated that 18-month-old Wistar females had a high (higher than males) level of the stress hormone cortisol. In a view of CR as a mild stressor, we could hypothesize that CR, introduced in aged females with high levels of cortisol, may induce additional stress that would overbalance the hormesis benefit that CR usually brings, thus

leading to an undesirable outcome. On the contrary, CR initiation at a younger age (15 months), might precondition animals to age-related stress that is about to happen a few months later. This could explain the difference in the cognition and frailty level after 3 months of CR noticed in 18- and 24-month-old females, but the lack of cortisol values is a limiting factor for this claim. However, the possible role of CR in preconditioning is supported by our previous studies on Wistar rats where CR applied before brain trauma suppressed inflammation and neurodegeneration (50); the proposed mechanism involves changed glucocorticoid signaling (51).

This study underpins a few scattered reports which appeared lately (reviewed in (18)) showing that in some cases CR could fail to induce beneficial effects. We further provide evidence to the opinion that there is an age at which CR is no longer effective or is unfavorable to a healthspan, at least according to some parameters. Unfortunately, a cross-sectional design of the study doesn't allow us to get a complete insight into the effects of different onset and duration of restricted feeding on longevity as, to be able to determine whether it prolongs life expectancy as well as healthspan, we would need a longitudinal study. Equally important, this study provides an insight into the sex differences in response to nutritional interventions. Given that the putative role of the sex hormonal status has been excluded by using females that were no longer in the reproductive stage of life, we suggest that there are other aspects that CR could target differently in males and females, for example, some specificities of their metabolisms (49) or the manner they cope with stress (52).

Due to the experimental concept that predicts sacrification of experimental animals at precisely defined time points, we miss the mortality rate data. This inputs certain limitations for proper validation (53,54) of this newly created physical-cognitive frailty score/index. However, we were following and detected the death rate during the experiment, until the

sacrification of the animals. During that period frailty index/score varied in accordance with the survival rate of the animals. As frailty decreases due to the EOCR treatment the number of survived animals increases, while LOCR increased frailty level and the death rate (eTable 6A and B). In addition, in eTable 6B individual time points of death during the experiment were shown. As CR was introduced step-wise, and since the CR was not extremely restrictive, but 60% of AL intake, we have no reason to think that deaths were caused by the reaction to the limited food amount. On contrary, the timing of death in 24 month-old AL group is quite similar to the timing of death in 24 months old LOCR group. This indicates that late-onset CR does not only increase frailty level, but in addition, is not effective in prolonging the lifespan.

Lastly, we suggest further improvement of existing frailty research and (diagnostic) tools. Not only physical but also psychological, cognitive, and social factors contribute to a multidimensional syndrome of frailty and need to be considered in its definition and treatment. Correlation between performances in two cognitive tasks with the frailty level determined in this manner emphasizes the benefits of including the cognitive component into frailty score/index calculation. As such it would offer a more comprehensive view of the health status of the organism. Further, identification of modifiable risk factors for cognitive frailty will contribute to the identification of high-risk individuals and help develop interventions to prevent cognitive decline in aging. On this ground, the results of this study impose great caution when introducing CR in humans. To achieve its favorable effect, the CR should be introduced in humans up to middle age (approximately corresponding to the age of 60).

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Conflict of interest

None reported.

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Figure Captions

Figure 1. Timeline of the experiment. The thick line represents the age of the animals during the experiment, while arrows mark the onsets and the duration of dietary regimens, EOCR and LOCR. Animals were sacrificed at 6-, 18- and 24-month of age (AL controls) or at 18 or 24 months (EOCR and LOCR); AL- *ad libitum*, EOCR- early-onset calorie restriction, LOCR- late-onset calorie restriction.

Figure 2. The behavioral profile of 6-, 18- and 24-month-old ad libitum (AL) and calorie restricted (EOCR and LOCR) female Wistar rats in a Y maze test. Spontaneous alternations **(A)**, number of 3rd arm entries **(B),** and time spent in 3rd arm **(C)**. Results are expressed as mean ± SEM for a 10-minute trial period. *p < .05 versus 6 AL group for the effect of aging, #p < .05 versus age-matching AL control for the effect of food regimen. $ Symbol implicates successful alternations at levels significantly above chance (50%) within a group. EOCR-early-onset calorie restriction, LOCR-late-onset calorie restriction.

Figure 3. Exploration ratio) in short-term **(A)** and long-term memory assessment **(B)** of 6-, 18- and 24-month-old ad libitum (AL) and calorie restricted (EOCR and LOCR) female Wistar rats. Results are expressed as mean ± SEM for 10 min choice trials. *p < .05 for the effect of aging. #p < .05 versus age-matching AL control for the effect of food regimen. EOCR- early-onset calorie restriction, LOCR- late-onset calorie restriction.

Figure 4. Frailty score (FS) and Frailty index (FI) of 6-, 18- and 24-month-old ad libitum (AL) and calorie restricted (EOCR and LOCR) female Wistar rats. Graphical representation of FS and FI during aging **(A, B)**, at 18 months under the influence of EOCR and LOCR versus age-matching AL control **(C, D),** and at 24 months under the influence of EOCR and LOCR versus age-matching AL control **(E, F)**. *p < .05 for the effect of aging. #p < .05 versus age-matching AL control for the effect of food regimen. EOCR- early-onset calorie restriction, LOCR- late-onset calorie restriction.

Figure 1

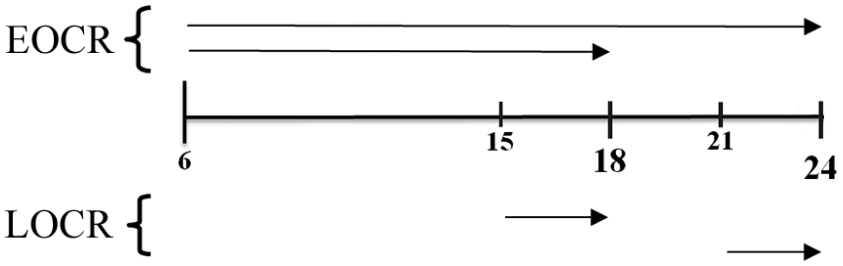

Figure 2

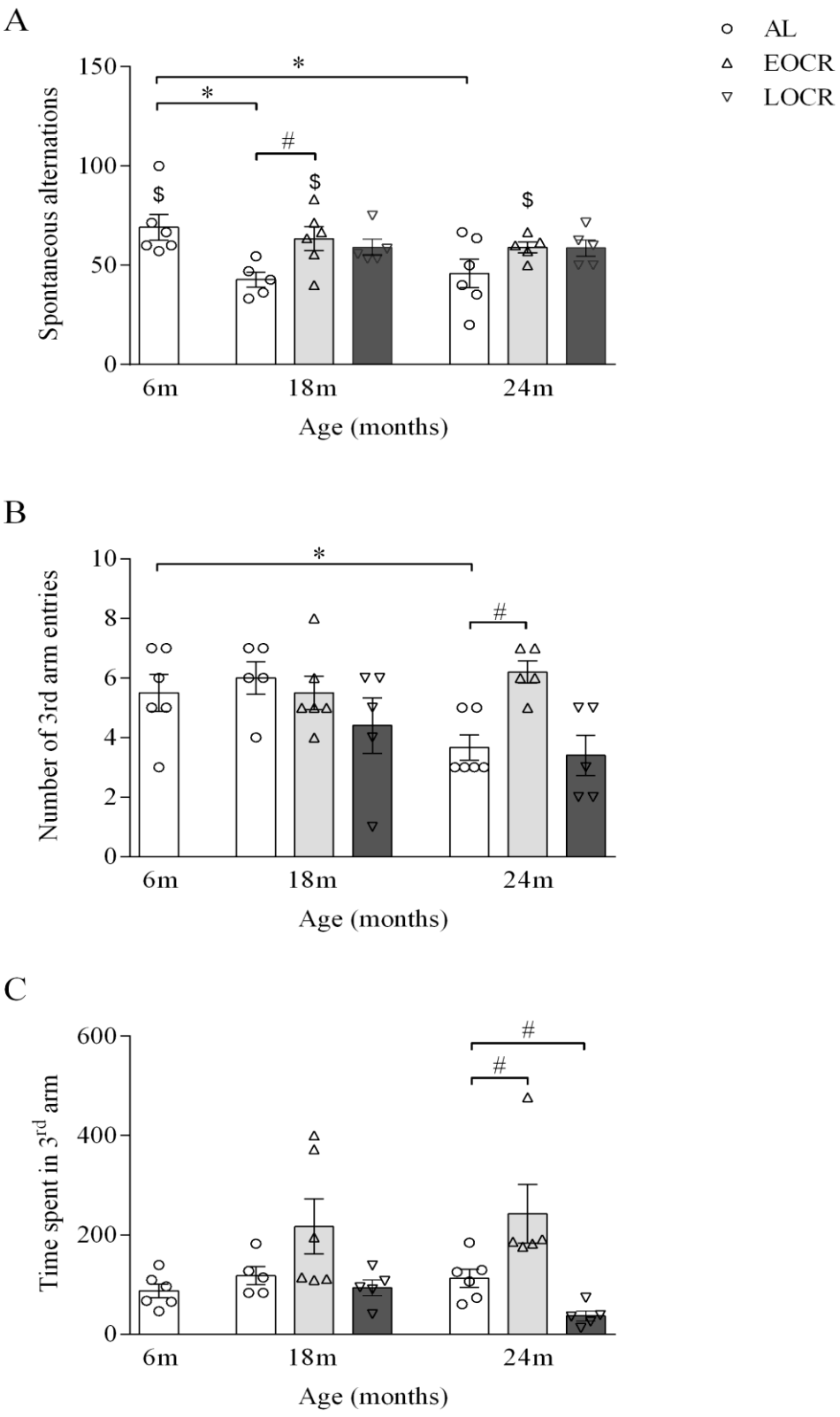

Figure 3

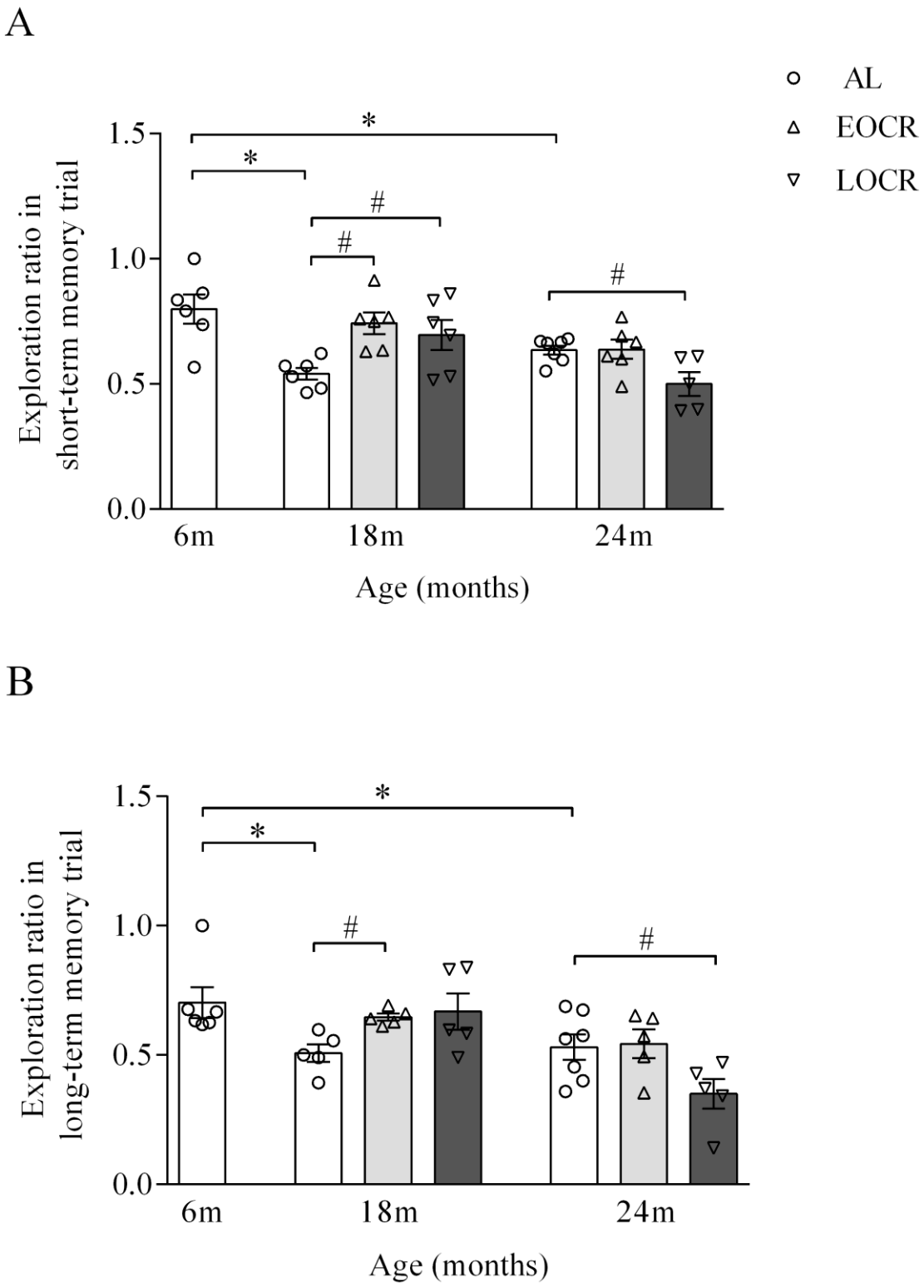

Figure 4