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Research article

The effect of late-life environmental enrichment on stress and anxiety: The role of sex and age-related differences in coping with aversive stimuli

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ABSTRACT

Anxiety significantly diminishes the quality of life in older adults, and the drugs used for its treatment often come with risky side effects. Non-pharmacological protocols could be valuable, but more research is needed in this area. Environmental enrichment induces positive effects on anxiety-like behavior in young and adult animals; whether the same happens in aged animals is still elusive. The aged brain undergoes changes that contribute to make it "fragile" and consequently even mild, potentially positive stimuli can trigger dyshomeostasis, worsening rather than ameliorating functioning. Here, by combining behavioral analysis and measurement of serum and brain corticosterone levels, we show that late-life environmental enrichment can induce eustress or distress, depending on sex and hypothalamic-pituitary-adrenal axis function. These findings pave the way for optimizing outcomes and minimizing undesired effects in the clinical setting, underscoring the need to overcome the limits of gender medicine and emphasizing the crucial role of individually tailored therapies.

1. Introduction

Anxiety affects 3.8% of the older population worldwide, according to the World Health Organization [1]. However, the actual prevalence among older adults could be significantly higher due to difficulties in diagnosis or cases being missed altogether [2,3]. Not only does anxiety cause disability and diminish the quality of life [4], but it also triggers a harmful cycle. Anxious older adults tend to avoid stressful situations, leading to increased social isolation and a higher risk for other psychiatric, neurological, and physical conditions [2]. This makes anxiety rightfully counted among the "geriatric giants" [5].

Several factors contribute to triggering anxiety onset during aging, such as atrophy in specific brain regions, epigenetic and hormonal changes, reduced telomere length, and deficits in the hypothalamic-pituitary-adrenal (HPA) axis. In particular, the impairment of the adaptive response to stressful stimuli due to HPA axis alterations enhances vulnerability and reduces resilience [6].

Benzodiazepines are commonly prescribed to treat anxiety, but they come with serious side effects in the older adults such as cognitive impairment, incontinence, falls leading to hip fractures, and fall-related deaths [2,7]. Additionally, older adults often have

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other medical disorders, which can lead to drug-drug interactions and worsen psychiatric symptoms [8]. As a result, there is a growing interest in non-pharmacological approaches for anxiety treatment [6,9,10], although more research is needed in this area. An overlooked issue is the importance of individually tailored protocols, as the response to treatments can be influenced not only by psychosocial factors but also by biological mechanisms which require careful consideration.

Environmental enrichment (EE) exerts well-established positive effects on anxiety-like behavior [11,12], potentially functioning as a stress inoculator that enhances resilience in animals throughout a hormetic approach [13]. However, most studies on EE have focused on young and adult animals, neglecting the distinctive characteristics and needs of aged animals, which necessitate thoughtful examination [14]. The aged brain undergoes changes that contribute to make it "fragile" [15,16] and consequently even mild, potentially positive stimuli can trigger dyshomeostasis, worsening rather than ameliorating functioning. In a previous study, we discovered that only certain animals benefit from a late-life enrichment protocol. Specifically, while some EE rats showed a decreased anxiety-like behavior when exposed to negative stimuli, other EE rats exhibited an anxious/fear reaction comparable to that of age-matched animals kept in standard housing [17].

Here, we explored the hypothesis that differences in HPA axis function, investigated by serum corticosterone levels, may discriminate animals responding to EE from those not responding. Additionally, the research investigates differences between sexes. The findings could pave the way for optimizing outcomes and minimizing undesired effects, especially with a view to translating the results into the clinical setting.

2. Methods

2.1. Animals

Male and female Sprague Dawley rats from the IRCCS INRCA colony were used. The rats were housed under standard environmental conditions with a 12-h light-dark cycle starting at 6 a.m.

At 17–18 months of age, the animals were transferred from the breeding facility to the experimental facility. After a one-week acclimation period, they were handled for 5 min per day for 5 consecutive days and familiarized with the test room for 10 min each day. Baseline measurements were obtained using the Open Field test (OF) and the Step-through Passive Avoidance test (PA). Following the baseline evaluation, the rats were semi-randomly assigned, considering their performance in the PA, to either continue standard housing (SH) or begin EE as in Balietti et al. [17]. Briefly, the EE cages (79 cm long, 52 cm wide, and 140 cm high) featured three floors, two balconies, and four flights. They were stocked with diverse items, including plastic tunnels, wooden gnawing sticks, toys, balls, paper nesting material, a running wheel, feeding boxes, and water bottles. To maintain a sense of novelty and encourage foraging behavior, these items were repositioned or replaced twice per week. This allows the rat to express a fundamental aspect of its ethogram, namely adaptation to a continuously changing natural environment.

Although the initial approach was always to keep together the highest possible number of previous cage mates, due to the required numerosity in the EE protocol, animals from different cages were inevitably housed together. This resulted, for male animals, in several episodes of fights to establish the hierarchy. The most evident event always occurred at cohort creation, but it could be repeated immediately after cleaning and reintroduction of the rats in the enriched environment. To mitigate the latter phenomenon, during cage cleaning, the animals were never separated. For the short period of time spent outside their experimental cage, they were housed together in another cage. This allowed for a progressive reduction/elimination in confrontations over the weeks. Indeed, no injuries (evident bites or scratches) were recorded, there were no episodes of isolation, and all the animals were able to eat and drink without limitations. SH adhered to the social requirements of the species by using cages (48 cm long, 37.5 cm wide, and 21 cm high) that facilitated both horizontal and vertical movements, while also allowing for isolation if needed. Additionally, wooden sticks and paper

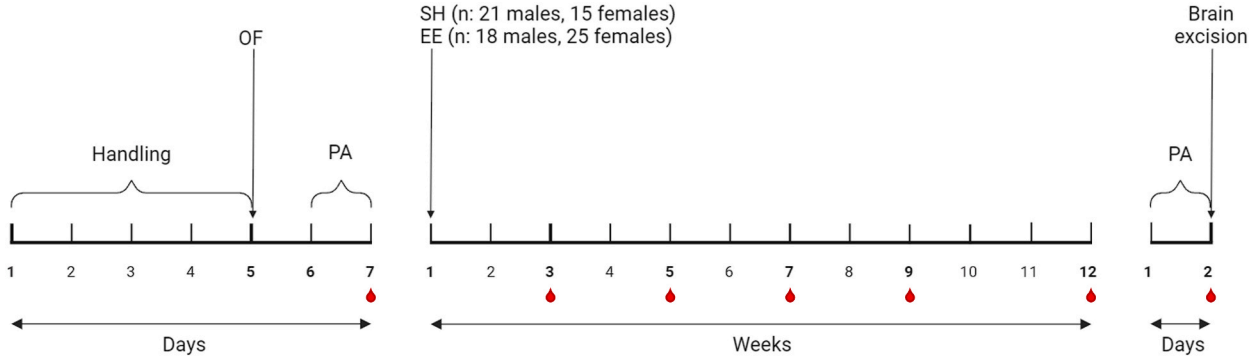

Fig. 1. The timeline of the experimental protocol. Rats were habituated to the researchers and the test room, followed by evaluations through the Open Field test (OF) and the Step-through Passive Avoidance test (PA). Subsequently, animals were semi-randomly subdivided to be housed for 12 weeks in standard (SH) or enriched (EE) conditions. Finally, a second PA was conducted before the animals were sacrificed. The red drops indicate blood withdrawals. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

nesting material were incorporated to fulfill the essential enrichment requirements for the well-being of the animals. Males were housed in groups of 8/10 in EE and 2 in SH, while females were housed in groups of 10/12 in EE and 3 in SH. The average body weight of the males was 710 ± 79 g, and that of the females was 375 [352–390] g. After 12 weeks, the surviving animals (EE: 18 males and 25 females; SH: 21 males and 15 females) were retested by PA (post-housing). In Fig. 1, a timeline of the protocol is provided.

The health status of the animals was continuously monitored following the protocol described by Phillips et al. [18]. Rats showing signs of poor general condition, following a thorough evaluation by a veterinarian to assess the irreversibility of their deteriorated well-being, were excluded from the study and subjected to humane endpoints (8 males and 5 females). Additionally, three males and two females experienced sudden mortality without exhibiting any discernible symptoms. All procedures were conducted in accordance with the European Union legislation (Directive 2010/63/EU) and were approved by the Italian Ministry of Health (code 35/2020-PR). Behavioral tests were performed between 1.30 p.m. and 2.30 p.m., and multiple separate cohorts of animals were tested for each experimental group to ensure the reproducibility of the results.

2.2. Open Field test

This test was used to assess the locomotor activity and anxiety-like behavior at resting condition [19,20] to exclude any potential bias resulting from the semi-random assignment of animals to the EE or SH group. The test was conducted in a rectangular arena measuring 60 cm (width) x 70 cm (length) x 35 cm (height). The arena was divided into two regions: center and border. Each rat was released at a corner and allowed to explore for 10 min. The arena was cleaned with 70% ethanol between rats to remove odor clues. EthoVision XT 10 software from Noldus (The Netherlands) was used to record total distance moved, mean velocity, number of rearings (unsupported and against the wall), time spent in the center, and number of fecal boli.

2.3. Step-through Passive Avoidance test

We utilized a shuttle box (57 cm long, 27 cm wide, and 30 cm high) with two equally sized compartments separated by a sliding

Table 1
Performances in the Open Field test of male and female rats successively subdivided in high latency and low latency by the Step-through Passive Avoidance test and semi-randomly assigned to environmental enrichment (EE) or standard housing (SH).

MALE RATS	EE		SH		
Total distance moved (cm)	high latency	2216 ± 823	high latency	1625 ± 695	[b] $p = 0.151$
	low latency	2080 ± 933	low latency	2129 ± 573	[c] $p = 0.900$
		[a] $p = 0.766$		[a] $p = 0.102$	
Mean velocity (cm/s)	high latency	3.70 ± 1.37	high latency	2.71 ± 1.16	[b] $p = 0.152$
	low latency	3.48 ± 1.55	low latency	3.55 ± 0.95	[c] $p = 0.899$
		[a] $p = 0.766$		[a] $p = 0.102$	
N of rearings	high latency	10.83 ± 8.03	high latency	5.33 ± 3.61	[b] $p = 0.134$
	low latency	9.17 ± 6.18	low latency	10.00 ± 9.09	[c] $p = 0.840$
		[a] $p = 0.663$		[a] $p = 0.243$	
Time spent at center (%)	high latency	10.65 ± 8.82	high latency	6.76 ± 10.52	[b] $p = 0.420$
	low latency	8.14 ± 7.26	low latency	5.66 [2.70–12.13]	[c] $p = 0.791$
		[a] $p = 0.559$		[a] $p = 0.235$	
N of fecal boli	high latency	1.50 [0.00–4.00]	high latency	1.83 ± 1.94	[b] $p = 0.933$
	low latency	3.33 ± 3.37	low latency	2.00 [0.00–6.00]	[c] $p > 0.999$
		[a] $p = 0.488$		[a] $p = 0.454$	
FEMALE RATS					
Total distance moved (cm)	high latency	2021 ± 939	high latency	2464 ± 618	[b] $p = 0.268$
	low latency	2092 ± 625	low latency	2434 ± 535	[c] $p = 0.227$
		[a] $p = 0.822$		[a] $p = 0.921$	
Mean velocity (cm/s)	high latency	3.63 ± 1.90	high latency	4.11 ± 1.03	[b] $p = 0.523$
	low latency	3.93 ± 1.30	low latency	4.16 ± 0.97	[c] $p = 0.676$
		[a] $p = 0.639$		[a] $p = 0.928$	
N of rearings	high latency	12.70 ± 9.40	high latency	19.00 ± 9.49	[b] $p = 0.178$
	low latency	17.87 ± 11.17	low latency	20.29 ± 7.16	[c] $p = 0.608$
		[a] $p = 0.241$		[a] $p = 0.774$	
Time spent at center (%)	high latency	6.22 ± 5.28	high latency	5.56 ± 3.98	[b] $p = 0.772$
	low latency	4.85 ± 3.14	low latency	6.58 ± 1.86	[c] $p = 0.065$
		[a] $p = 0.421$		[a] $p = 0.547$	
N of fecal boli	high latency	0.00 [0.00–3.00]	high latency	0.00 [0.00–6.00]	[b] $p = 0.798$
	low latency	0.00 [0.00–0.00]	low latency	0.00 [0.00–4.00]	[c] $p = 0.682$
		[a] $p = 0.117$		[a] $p = 0.856$	

[a] *p*, high latency *vs*. low latency.
[b] *p*, EE high latency *vs*. SH high latency.
[c] *p*, EE low latency *vs*. SH low latency. Numerosity: high latency and low latency males assigned to EE, n = 12 and n = 6; high latency and low latency males assigned to SH, n = 6 and n = 15; high latency and low latency females assigned to EE, n = 10 and n = 8; high latency and low latency males assigned to SH, n = 8 and n = 7.

door (Ugo Basile srl, Italy). One compartment was illuminated, while the other remained dark. On the first day, the rats were placed in the illuminated chamber, and once all four paws entered the dark compartment, an electric foot shock was administered (0.8 mA for males and 0.6 mA for females, lasting 2 s). Rats that failed to vocalize or jump were excluded (n = 1) [21]. The intensity of the electric shock applied was higher in males because Sprague Dawley rats tend to significantly increase body fat mass with aging, and this mass is among the tissues with greater resistance. Therefore, while maintaining values capable of causing only a sensation of discomfort without causing any harm, we differentiated the amperage values between the two sexes. After 24 h (test phase), the rats were again placed in the illuminated compartment, and the time it took for the animal to enter the dark chamber (latency) was recorded. If the rat remained in the illuminated compartment for the duration of 300 s, the test was considered complete [22]. Rats with latencies exceeding 200 s or falling below 100 s were categorized as high latency or low latency, respectively. These specific cutoff values were chosen as they effectively dichotomized the rats, with nearly all falling above or below these thresholds. Rats with intermediate performances were excluded from the analysis to prevent potential classification biases (n = 2) [17]. The apparatus was cleaned with 70% ethanol between rats to remove odor clues.

PA, which is a conditioned-based task, was selected to categorize the rats over other tests based upon naturally occurring anxiety-like behavior, such as the Elevated Plus Maze, because, in assessing aversive memory, it encompasses anxiety-like behavior and fear under stressful conditions [23]. This optimizes the possibility of identifying those animals that, under the mild stressor effect of the EE, learn coping strategies enabling them to effectively manage potentially adverse situations. Specifically, PA functions by inhibiting an innate instinct -the preference for darkness inherent in rats as nocturnal animals- when it is associated with a negative stimulus. Therefore, in this test, anxiety-like behavior or fear manifests as passive defensive conduct, with the rats choosing to stay in the illuminated chamber. This behavior is typically accompanied by a pronounced increase in the neuroendocrine response, including the activation of the HPA axis and an elevation in glucocorticoid secretion [24]. Entry into the dark compartment, on the other hand, could be the result of either the failure to remember the negative stimulus received the previous day (*i.e.*, mnemonic impairment) or a reduction in anxiety-like behavior triggered by stressors [17]. The trend of corticosterone serum levels, together with the different behavior of rats in the experimental compared to those in the control group, suggests whether a low latency performance is due to one condition or the other (see captions to figures for details). So, PA is the preferred choice to verify whether late-life interaction with enriched housing can still determine a hormetic effect and whether it is applicable to all aged rats or not.

2.4. Concentration of serum corticosterone

Since in nocturnal animals the HPA axis has its peak reactivity to stimuli during the light period of the day [25], we collected blood samples between 2 p.m. and 4 p.m. Blood sampling was always conducted on different days from EE cage cleaning to avoid any potential influence on corticosterone levels of acute, albeit transient, rehousing stimulation.

Blood samples were drawn under deep anesthesia with isoflurane while the rats maintained spontaneous breathing. Approximately 800 μL of blood were collected from the tail vein of each rat at various time points throughout the study. The first blood sample was taken 30/45 min after the test phase of the initial PA (time point 1) [6]. Subsequent blood samples were collected every 2 weeks during the first 8 weeks (time points 2, 3, 4, and 5), followed by a 3-week interval before the next blood sample to minimize vessel trauma (time point 6), and a final blood sample was obtained at post-housing, 30/45 min after the test phase of the second PA (time point 7). The blood samples were incubated at 37 °C for 1 h and then centrifuged at 2000×*g* for 20 min at 4 °C to separate the serum. Serum corticosterone concentrations were determined using a commercially available ELISA kit (# EIACORT, ThermoFisher SCIENTIFIC) following the manufacturer's instructions. The samples were tested in duplicate, and the results were expressed as μg/dL.

Blood sampling was carried out following the guidelines provided by NC3Rs to ensure that multiple draws did not compromise the well-being of the animals [26]. The only deviation was the use of anesthesia instead of a restraint method. Although the rats were fully accustomed to manipulation by the operators, this choice provided greater certainty in preventing potential stress that could affect corticosterone levels. Furthermore, anesthesia lasted only a few minutes per animal and guaranteed a swift and complete recovery immediately afterward.

2.5. Levels of corticosterone in hippocampus and amygdala

At time point 7, all animals were sacrificed. Since the PA primarily relies on the dorsal hippocampus and basolateral amygdala for

Table 2
Number of high latency and low latency rats after the first (baseline) and the second (post-housing) Step-through Passive Avoidance test.

	BASELINE	*males*	*females*	POST-HOUSING	*males*	*females*
EE	high latency	n = 12	n = 10	high→high latency	n = 5	n = 5
				high→low latency	n = 7	n = 5
	low latency	n = 6	n = 15	low→low latency	n = 6	n = 8
				low→high latency°	**n = 0**	**n = 7**
SH	high latency	n = 6	n = 8	high→high latency	n = 6	n = 8
				high→low latency	n = 0	n = 0
	low latency	n = 15	n = 7	low→low latency	n = 8	n = 7
				low→high latency°°	**n = 7**	**n = 0**

In bold the subgroups that evidenced sex-related differences: $^{\circ}p = 0.040$, $^{\circ\circ}p = 0.029$ (χ^2 test).

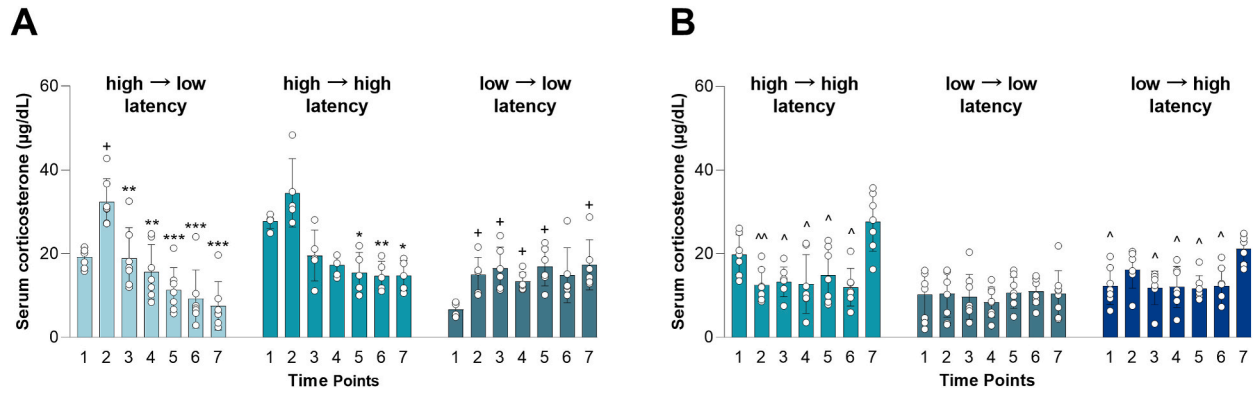

Fig. 2. High latency males took advantage of environmental enrichment (EE) through a hormetic stimulation. A) In EE animals initially classified as high latency, there was an increase in serum corticosterone levels during the first two weeks (time point 2), followed by a progressive decrease that continued until time point 7 in rats that became low latency. Conversely, in rats that remained high latency, the corticosterone levels reached a steady state trend from time point 4 to time point 7, thus even under the aversive stimulation of the post-housing Step-through Passive avoidance test (PA). In EE animals classified as low latency at both PAs, serum corticosterone levels exhibited a significant increase during the first two weeks and remained elevated throughout the entire experimental period. B) Under standard housing, low latency rats displayed a stable pattern of serum corticosterone levels throughout the entire experimental period, except for an increase at time point 7 for those that became high latency. High latency animals that remained high latency had higher serum corticosterone levels induced by the two PAs.
Distinguishing between animals classified as having low latency at post-housing, whether they exhibit memory impairment or decreased anxiety-like behavior, depends on the trend of corticosterone serum levels, along with the differences between rats in the experimental group and those in the control group. Animals with low latency at both tests reasonably had cognitive deficits: in SH, the functioning of their hypothalamic-pituitary-adrenal (HPA) axis remained unchanged, while in EE, it was dysregulated, a condition, the latter, that certainly cannot exert anxiolytic effects but can further damage cognitive performance. Conversely, animals in the EE that initially exhibited high latency but converted to low latency have likely developed effective coping strategies (*i.e.*, a decrease in hormone production), indicating a progressive adaptation of the HPA axis. This suggests that their performance in the task is attributable to reduced anxiety-like behavior.
$+\ p < 0.05$ [*vs.* time point 1]; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ [*vs.* time point 2]; $\hat{}\ p < 0.05$, $\hat{}\hat{}\ p < 0.01$ [*vs.* time point 7].

its functioning [27], the hippocampi and amygdalae were excised from each rat. The excised brain regions were then weighed and homogenized in PBS at a ratio of 1 mL per 100 mg of tissue. The homogenates were stored at −20 °C overnight. The following day, the samples underwent two cycles of freezing and thawing. Afterward, the homogenates were centrifuged at 5000×*g* at 4 °C for 5 min, and the resulting supernatants were collected and maintained at −80 °C until further use.

To determine the concentration of corticosterone in the brain tissue, a commercial ELISA kit (#CSB-E07014r, CUSABIO) was employed, following the instructions provided by the manufacturer. The samples were tested in duplicate, and the results were expressed as ng/mg of brain tissue.

2.6. Statistical analysis

To assess the normality of the variables, the Kolmogorov-Smirnov test was conducted. The results of the analysis were expressed as either the mean ± standard deviation or the median with interquartile range, depending on the distribution of the data. For the comparisons of performances in the OF, either the unimpaired t-Student test or the Mann-Whitney test was used. In the case of serum molecules, comparisons were made using either a one-way ANOVA for repeated measures or Friedman's tests; *post hoc* comparisons were conducted using Tukey's test and Dunn's test. The comparison among cerebral corticosterone levels was performed by ordinary one-way ANOVA, Kruskall-Wallis or unpaired t-Student test using Holm-Sidak's test and Dunn's test for *post hoc* comparisons. A significance level of $p < 0.05$ was considered statistically significant for all analyses.

In adherence to the REDUCTION principle of the 3Rs, we implemented a 'rolling evaluation' of the sample size necessary to achieve a minimum power of 80% for our primary outcome. Our objective was to investigate the hormetic effect of EE on anxiety-like behavior in aged rats. To test this hypothesis, we utilized the progressive reduction of serum corticosterone levels during the period of EE housing. Building on findings from the previous study [17], which suggested that animals classified as high latency at baseline were the only beneficiaries of EE, we compared corticosterone levels at time point 2 (representing the maximum effect as a mild stress inoculator per the protocol) and at time point 7 (when animals were assessed after an aversive stimulus). The resulting difference was employed to calculate the effect size. We concluded the enrollment of new animals after reaching a sample size of 12 high latency rats for males and 10 high latency rats for females to house in EE. With an α-error set at 0.05, effect sizes of 0.87 for males and 0.88 for females and employing a one-tailed paired t-Student test focused solely on the hypothesis of hormone down-regulation, the β-error values were determined to be 0.88 and 0.82 for males and females, respectively (calculated using G*Power software).

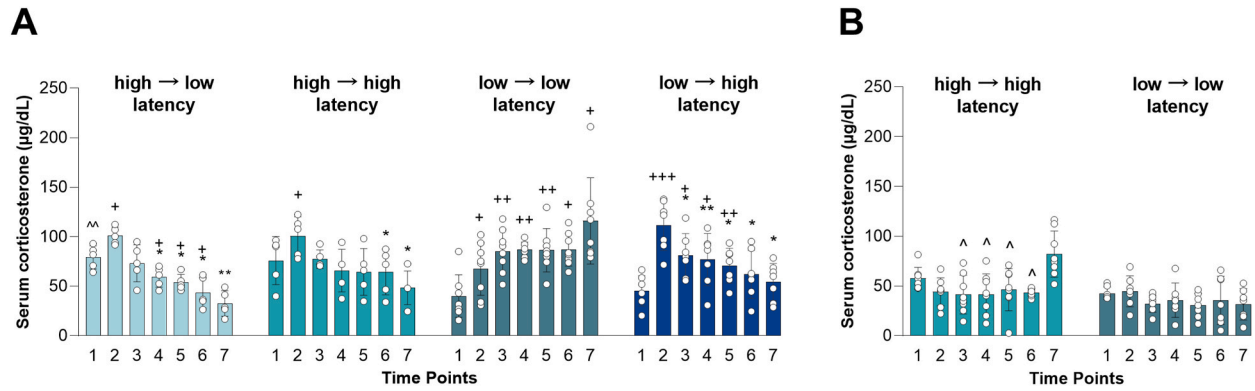

Fig. 3. High latency and a subgroup of low latency females coped with environmental enrichment (EE)-related stressful stimuli. A) During EE, female animals initially classified as high latency experienced an increase in serum corticosterone levels during the first 2 weeks, followed by a progressive decrease. Specifically, high latency animals classified as low latency at post-housing reached significance compared to time points 1 and 2 starting at time point 4, while high latency animals that remained high latency reached significance only compared to time point 2 at time point 6. In contrast, female rats initially classified as low latency displayed opposite responses. Those animals that converted to high latency showed a significant increase in serum corticosterone levels from time point 1 to time point 2 but then progressively returned to the time point 1 value. Conversely, those animals that remained low latency exhibited significantly higher serum hormone concentrations compared to time point 1 from time point 2 onward. B) High latency females that remained high latency maintained under standard housing had significantly higher serum corticosterone levels triggered by both the Step-through Passive Avoidance tests compared to the other time points, while low latency animals that remained low latency maintained a steady-state pattern for the entire period.
Distinguishing between animals classified as having low latency at post-housing, whether they exhibit memory impairment or decreased anxiety-like behavior, depends on the trend of corticosterone serum levels, along with the differences between rats in the experimental group and those in the control group. Animals with low latency at both tests reasonably had cognitive deficits: in SH, the functioning of their hypothalamic-pituitary-adrenal (HPA) axis remained unchanged, while in EE it was dysregulated, a condition, the latter, that certainly cannot exert anxiolytic effects but can further damage cognitive performance. Conversely, animals in the EE that initially exhibited high latency but converted to low latency have likely developed effective coping strategies (*i.e.*, a decrease in hormone production), indicating a progressive adaptation of the HPA axis. This suggests that their performance in the task is attributable to reduced anxiety-like behavior.
$+\ p < 0.05$, $++\ p < 0.01$ [*vs.* time point 1]; $*p < 0.05$, $**p < 0.01$ [*vs.* time point 2]; $\hat{}\ p < 0.05$, $\hat{}\hat{}\ p < 0.01$ [*vs.* time point 7].

3. Results

High latency and low latency rats performed comparably in the OF, regardless of their assignment to EE or SH, and irrespective of sex (Table 1). This indicates that the performance in the PA was unlikely influenced by any physical or behavioral abnormalities in the rats and the assignment to the housing conditions was reasonably unbiased. Table 2 summarizes the subdivision of animals before and after the housing protocols based on their performance in the two PAs. Rats initially classified as low latency showed sex-related differences: all SH females remained low latency, while 47% of SH males became high latency ($p = 0.029$); all EE males remained low latency, while 47% of EE females converted to high latency ($p = 0.040$).

To verify if animals retained memory of the first PA (a possible carry-over effect due to the repetition of the test), baseline and post housing latencies at first day of the task were compared: animals in all groups exhibited no significant differences, except for SH low latency males that converted to high latency and SH high latency females that remained high latency. Their latencies significantly increased (18.00 s ± 14.33 s *vs.* 51.75 s ± 25.64 s, $p = 0.015$; 15.56 s ± 11.69 s *vs.* 35.97 s ± 14.70 s, $p = 0.033$; paired t-Student test) as a consequence of the re-exposure to the apparatus, where they remembered having previously received the foot shock. These findings provide evidence that there was no recall phenomenon observed in any animal housed in EE when re-exposed to the PA apparatus. This implies that the observed performances can be exclusively attributed to the housing protocol and that the baseline test, crucial for any potential pre-classification of animals that will benefit from EE, does not interfere with the experimental design.

3.1. *Based on the function of HPA axis, males and females react differently to EE*

EE male animals initially classified as high latency showed a significant increase in serum corticosterone levels from time point 1 to time point 2, followed by a gradual decrease until time point 7 in those that converted to low latency and until time point 4 in those that remained high latency, which then reached a *plateau*. In contrast, EE low latency males displayed a significant increase in serum corticosterone levels from time point 1 to time point 2 onward (Fig. 2A). SH high latency male rats had higher serum corticosterone levels after both PAs, with significance compared to the other time points observed only at retest. SH low latency males that remained low latency maintained a consistent and stable trend throughout the 12-week housing protocol, while low latency rats that converted to high latency significantly increased the serum corticosterone level at time point 7 (Fig. 2B).

Female rats initially classified as high latency or low latency at baseline and high latency at post-housing displayed similar patterns of serum corticosterone levels during EE, characterized by an increase from time point 1 to time point 2, followed by a progressive

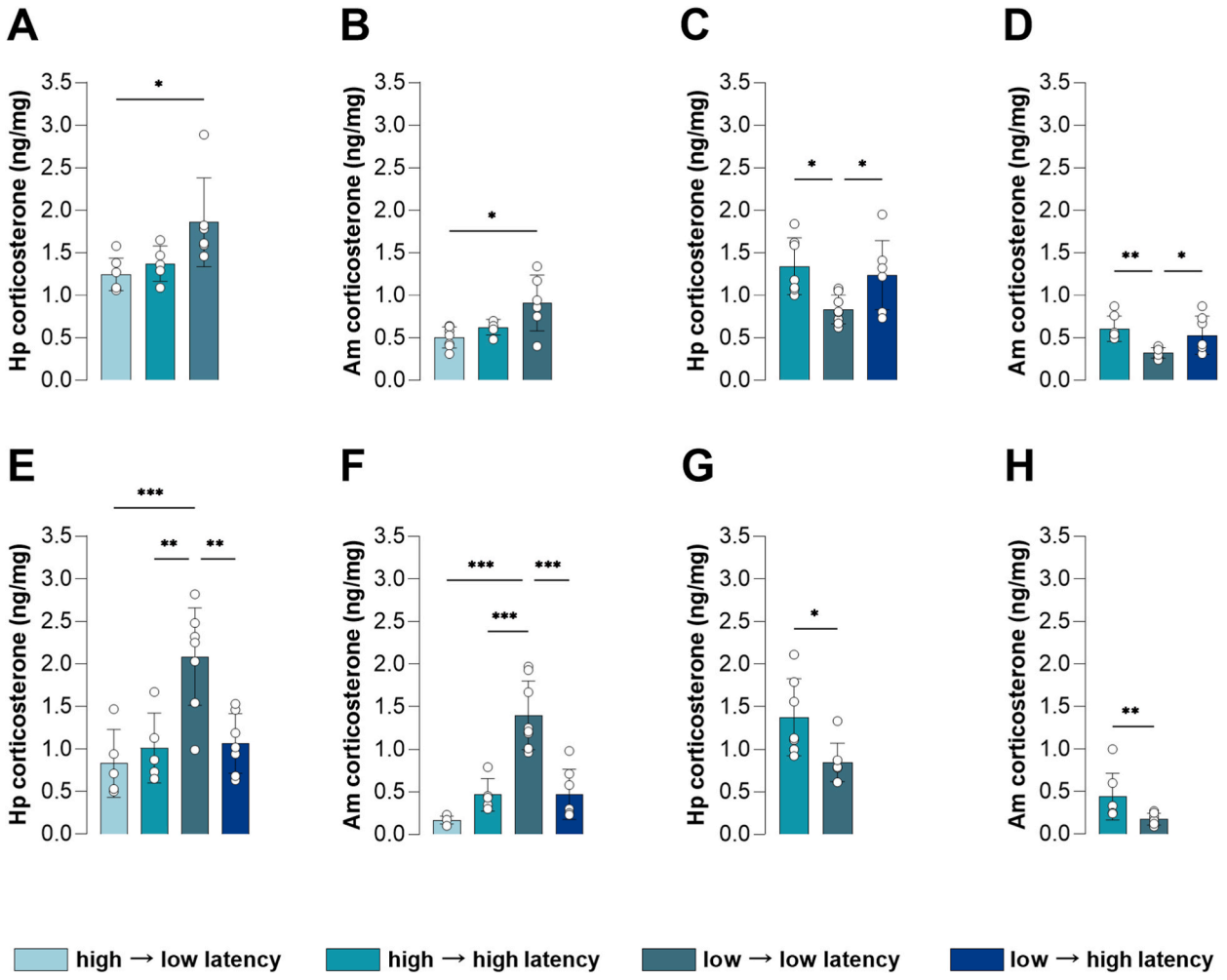

Fig. 4. Cerebral levels of corticosterone mirror the serum amounts in male and female rats. Low latency males that underwent environmental enrichment (EE) and remained low latency had significantly higher corticosterone than animals initially classified as high latency that converted to low latency, both in hippocampus (A) and amygdala (B). Under standard housing (SH), low latency males that remained low latency had significantly lower corticosterone compared to the other animals both in hippocampus (C) and amygdala (D). EE high latency females and low latency females that became high latency had significantly lower corticosterone than low latency animals that remained low latency in both hippocampus (E) and amygdala (G). SH high latency females had higher corticosterone in hippocampus (H) and amygdala (F) compared to low latency rats. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

decrease. High latency females that converted to low latency exhibited the most pronounced trend. On the other hand, low latency animals that remained low latency had a significant increase in serum corticosterone levels from time point 2 onwards compared to time point 1 (Fig. 3A). SH females maintained a stable serum corticosterone level throughout the 12 weeks, except for a significant increase at retesting in high latency rats that remained high latency (Fig. 3B).

Supplementary Table 1 reports detailed information regarding the statistical analysis outcomes (*i.e.*, F and Fr, DF as well as the *p* values).

Hence, males behaved differently from females during EE. All animals experienced increases in serum corticosterone due to the chronic stress induced by the housing protocol and all high latency animals acquired coping skills following the initial weeks. Nonetheless, while high latency females that remained high latency exhibited serum corticosterone akin to their basal values, the corresponding male group showed a less pronounced reduction. Moreover, while none of low latency males adapted to EE, about half of the females initially classified as low latency, after an early strong response of the HPA axis, had a progressive, low-grade ability to reduce serum corticosterone. Finally, none of the SH low latency females changed their classification at retest.

However, regardless of sex-specific differences, a common synthesis of the EE effect can still be found. Except for animals classified as low latency at baseline that maintained that classification at post-housing, all other groups benefited from EE. During the aversive situation of the second PA, they maintained stable corticosterone levels (*i.e.*, did not experience a peak as animals in the SH did), suggesting an increased capacity to effectively manage potentially negative stimuli.

It may be interesting to highlight that even in the senescent phase, female rats maintain a more robust neuroendocrine response to stress, with blood concentrations of corticosterone higher than those of male rats. Besides, it is known that beyond the influence of gonadal hormones on the HPA axis, there are other factors affecting its differential functioning in the two sexes. The topic goes beyond the objectives of our research but is addressed in detail by Heck and Handa [28].

3.2. Cerebral levels of corticosterone mirror the serum levels in male and female rats

Regarding the corticosterone levels in hippocampus and amygdala, both male and female animals showed patterns that mirror those found in serum (Fig. 4).

4. Discussion

Our study provides evidence that EE in aged rats can act as either a beneficial or harmful stressor. Indeed, while some animals acquired proficient coping mechanisms in response to EE through a hormetic phenomenon, leading to a reduction in anxiety-like behavior when exposed to aversive situations, others undergo continuous hyperactivation of the HPA axis. Among males initially classified as high latency coping strategies were employed, while males classified as low latency experienced distress conditions. Among females, both high latency animals and a subgroup initially classified as low latency exhibited the ability to mitigate the impact of stress, while others experienced continuous negative stimulation. Serum corticosterone emerged as a reliable biomarker, supporting the role of the HPA axis in distinguishing responder and non-responder aged rats to EE. Importantly, the serum concentrations correlated with cerebral levels, highlighting a crucial characteristic of peripheral biomarkers -their ability to reflect the function of the target organ.

During aging, the HPA axis undergoes deficits that affect the capacity to properly respond to physical and psychological threats. However, as with all age-related changes, HPA axis dysfunctions are not uniform [6]. This heterogeneity can significantly impact the response to negative stimuli in aged subjects, as demonstrated by studies on several species, including non-human primates, which are animals with cognitive and behavioral patterns most similar to those of humans, even in terms of anxiety [29]. Developing protocols that can enhance resilience by acting on the HPA axis, stimulating potential functional reserves that would otherwise remain “silent”, represents a field of study with considerable translational value.

Since EE reduces anxiety-like behavior and fearfulness by affecting the HPA axis [30], it is important to differentiate, prior to or during the protocol, animals with a preserved or impaired HPA axis functioning. This procedure guarantees that non-responders either abstain from the housing condition (all low latency males) or are promptly removed from it upon the onset of negative consequences (low latency females that remained low latency). Indeed, prolonged, elevated concentration of glucocorticoids negatively affects health, predisposing to sleep problems, cognitive impairment, immunosenescence, and frailty [31–34].

Regarding the influence of sex, females seem to better behave under EE, as all high latency and a subgroup of low latency rats adopted effective coping strategies while only high latency males did so. These results are in line with the findings of Bowman et al. [35], who showed that chronic stress has an anxiolytic effect in aged female rats but increases anxiety in aged male animals. The results of the SH groups support the notion that males can be more anxious. About half of the males initially classified as low latency had a significant increase in serum corticosterone triggered by the post-housing PA, because of the recall of the aversive protocol during the conditioning day. This suggests that some low latency males, but no low latency females, suffer from repeated acute stress, increasing their anxiety-like behavior and ultimately fear, as proved by their conversion to high latency. The anxiolytic effect of estrogens has been extensively documented [36,37]. Additionally, there is evidence that under chronic mild stress, estrogen exerts an anxiolytic-like action through activation of either the receptor β (Erβ) and/or the G protein-coupled receptor, with the modulatory effects of estrogen on the GABAergic system likely mediated through Erβ [38]. Although gonadal synthesis progressively declines with aging, recent research suggests that the brains of female rats may adeptly compensate for this decline [39], thereby preserving protective effects also during senescence. However, it cannot be excluded that EE induces a higher level of stress in male rats *per se* [40]. The instinct to define the hierarchy and the inevitable confrontations that this entails may have certainly made the management of the experimental setting more challenging for males compared to females. However, it is important to emphasize that we carefully avoided overcrowding, by considering the weight of the animals and not just the number [14]. In addition, once the cohort was formed, the animals were never separated during the 12 weeks, which progressively stabilized the hierarchy. These approaches allowed us to minimize difficulties related to the territoriality of male subjects as proved by the absence of aggressions causing injuries, the limited temporal duration of conflicts, and the absence of deterioration in well-being due to opposition in foraging.

In conclusion, in aged rats EE can elicit either eustress or distress, depending on sex and HPA axis function, as proved by serum corticosterone modulation. As the findings from EE research hold potential implications for humans and for approaches capable to enhance the cognitive reserve [41], our results could pave the way for increasingly individually tailored protocols. It becomes evident that there is a crucial necessity to evaluate each subject from a perspective that extends beyond gender medicine and embraces the broader concept of personalized therapy.

Data availability statement

Data will be made available on request.

Ethics approval and consent for publication

All procedures were approved by the Italian Ministry of Health (code 35/2020-PR) in accordance with the European Union legislation (Directive 2010/63/EU).

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CRediT authorship contribution statement

Marta Balietti: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Elisa Principi:** Investigation, Data curation. **Luca Giacomini:** Methodology, Investigation. **Belinda Giorgetti:** Methodology, Investigation. **Fiorenzo Conti:** Writing – review & editing, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nothing to declare. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32021.

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