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The ratio of prematurely aging to non-prematurely aging mice cohabiting, conditions their behavior, immunity and lifespan

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ABSTRACT

Adult prematurely aging mice (PAM) show behavioral deterioration, premature immunosenescence and increased oxidative stress, impairments that are associated with their shorter lifespan, compared to the corresponding exceptional non-prematurely aging mice (ENPAM). When PAM live in a predominantly ENPAM environment (2/5, respectively) they exhibit an improvement of immunity and redox state in their spleen and thymus leukocytes, and an increased lifespan. Nevertheless, it is unknown if other PAM/ENPAM ratios could affect behavioral and peritoneal leukocyte functions of PAM and change their lifespan. ENPAM and PAM were divided into the following groups: C-ENPAM (8 ENPAM in the cage); C-PAM (8 PAM in the cage); ENPAM > 50% and PAM < 50% (5 ENPAM/2 PAM in each cage); ENPAM = 50% and PAM = 50% (4 ENPAM/4 PAM in each cage), and PAM > 50% and ENPAM < 50% (5 PAM/2 ENPAM in each cage). After two months, mice were submitted to a battery of behavioral tests. Several functions and oxidative stress parameters were then assessed in their peritoneal leukocytes. Animals were maintained in these conditions to analyze their lifespan. The results showed that PAM > 50%, PAM = 50% and PAM < 50% exhibited better behavioral responses, immunity and redox states in their peritoneal leukocytes than C-PAM. This improvement was higher when the number of ENPAM in the cage was increased, with most of the parameters in PAM < 50% reaching similar values to those in C-ENPAM, and an increased lifespan. However, ENPAM that cohabited with PAM showed, in general, an impairment of parameters studied. In conclusion, the PAM/ENPAM cohabitation ratio is relevant to behavior and immunity.

1. Introduction

Aging is associated with a general and progressive deterioration of all physiological system functions, especially those of the regulatory systems (nervous, immune and endocrine systems), leading to a loss of homeostasis and, consequently of health. This age-related decline seems to be associated with an imbalance between oxidant compounds and anti-oxidant defenses, in favor of the first, which constitutes a chronic oxidative stress (De la Fuente and Miquel, 2009). In this context, the nervous system shows age-related alterations in neurons and in the synthesis and release of neurotransmitters, which are associated with a decline of many functions. In fact, chronologically old individuals show an impairment of sensorimotor abilities, motor capacities and altered emotional response, as higher anxiety-like behaviors (Brenes et al., 2008; Greenwood and Parasuraman, 2003; Krampe, 2002; Li and Lindenberger, 2002). With respect to the immune system, its age-related changes or immunosenescence seem to be the consequence of the loss in leukocytes of their capacity to regulate the oxidant and inflammatory compounds that they produce in order to carry out an immune response. Thus, an increase of the chronic oxidative stress of the organism is generated (De la Fuente and Miquel, 2009; Hazeldine and Lord, 2015; Salminen et al., 2008; Tu and Rao, 2016; Vida et al., 2017; Weyand and Goronzy, 2016). This affects innate and acquired immune responses, leading to a greater susceptibility to infectious diseases, autoimmune processes and cancer (De la Fuente and Bauer, 2016; De Martinis et al., 2006; Gruver et al., 2007). In fact, there is a link between the redox state of the immune cells, the function capacity of these cells and the lifespan of an individual (De la Fuente and Miquel, 2009).

One of the characteristics of aging is its high heterogeneity, which is shown as a different rate of aging in each individual, regardless of chronological age. This leads to the concept of biological age, which

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better estimates the physiological state, health, vitality and remaining life expectancy of each subject (Borkan and Norris, 1980; Bulpitt et al., 2009). In this context, several immune functions have recently been proposed as markers of biological age and predictors of longevity (Martínez De Toda et al., 2016). Therefore, to corroborate these biological markers and considering the long-life expectancy of humans, animal models of premature aging are a useful approach. In this sense, we have developed a premature aging model based on the behavioral response shown by chronologically adult mice exposed to a new environment, such as a T maze. In fact, adult animals showing slower locomotion and higher time to cross the intersection of the T-maze than their counterparts of the same sex and chronological age were considered prematurely aging mice (PAM). Those mice with a lower time performing the T-maze were considered exceptional non-PAM: ENPAM). PAM exhibit an excess of reactivity to stress, with higher freezing and grooming behaviors as well as a premature immunosenescence and higher oxidative stress, in comparison to ENPAM (Martínez De Toda et al., 2016, 2020; Viveros et al., 2007). All these impairments contribute to a shorter lifespan in the PAM than in their ENPAM counterparts, confirming their higher biological age (Martínez De Toda et al., 2016; Guayerbas et al., 2002a).

Social species, such as humans and rodents, are profoundly influenced by their social environment, which affects their behavioral and physiological responses and is essential for survival and reproductive success. In this context, most studies have aimed at examining the effects of a negative environment on health. Thus, loneliness in humans and social isolation in mice cause impairments of behavioral responses and immune system functions, which result in a decreased lifespan (Arranz et al., 2009; Bugajski, 1999; Cruces et al., 2014; Cohen et al., 2007). Negative social influences, such as that produced by cohabitation with sick individuals, have also been reported. In fact, when healthy mice cohabited with non-transmissible sick individuals, the first showed detrimental effects in their regulatory systems, exhibiting behavioral and immune alterations (Morgulis et al., 2004; Palermo-Neto and Alves, 2014). Furthermore, a previous report has described the induction of scratching behavior and dermatitis in various strains of healthy mice by cohabitation with animals showing spontaneous atopic dermatitis (Hashimoto et al., 2006). By contrast, the existence of strong social networks may profoundly influence the physiological responses, this being positively correlated with health (Devries, 2002; Seeman and Crimmins, 2001; Uchino, 2006), especially in chronological and premature aging. In agreement with this, a previous study has described that old mice can improve their behavioral responses and peritoneal leukocyte functions in cohabitation with a predominantly adult environment, showing longer lifespans than old control mice (Garrido et al., 2018a). Furthermore, premature immunosenescence in peritoneal leukocytes from tyrosine hydroxylase haploinsufficient mice (TH-HZ), a genetic alteration proposed as a premature aging model (Garrido et al., 2018b), may be avoided when these animals cohabit in a predominantly wild type environment (Garrido et al., 2017). Also, spleen and thymus leukocytes from PAM, after cohabiting in a predominantly ENPAM environment, show a slowing down of their immunosenescence and oxidative stress (Garrido et al., 2019a, 2019b). Thus, this kind of cohabitation seems to be capable of improving the regulatory system functions in the context of aging, exerting positive effects on longevity, when in the cage the proportion of aged mice is lower than non-aged mice. However, this social environment also exerts negative consequences on adult mice. In fact, when five adult mice were co-housed with two chronologically old mice, the adults presented several behavioral abnormalities and premature immunosenescence, after two months of cohabiting, although without consequences on their lifespans (Garrido et al., 2018a). Similar results have been described in wild type mice and ENPAM that cohabited with TH-HZ animals and PAM, respectively, in this proportion (Garrido et al., 2017; Garrido et al., 2019a, 2019b). Nevertheless, the potential effects presented by other ratios in the number of aged/non-aged animals in the same cage, are not known. Therefore, the aim of this work was to determine if the cohabitation of different PAM/ENPAM ratios ($> 50\%$; $= 50\%$ or $< 50\%$) could show positive effects on behavior and immune function parameters as well as on the lifespan of both PAM and ENPAM and whether these effects are a gradual to the number of PAM/ENPAM in the cage.

2. Methods

2.1. Animals

Two hundred female outbred CD1 mice (Janvier, France) between thirty-two to thirty-three weeks of age were purchased. They were housed seven to eight per cage and maintained with *ad libitum* access to food and tap water under light (12 h light/dark cycle; lights off at 8:00 AM), temperature (22 ± 2 °C) and humidity (50–60%) controlled conditions. We used this strain due to its higher genetic heterogeneity than inbred strains. Moreover, we have performed the majority of the previous studies with PAM and ENPAM using the CD1 strain (Guayerbas et al., 2002a; Guayerbas and De la Fuente, 2003; Martínez De Toda et al., 2016; Viveros et al., 2007). Diet was in accordance with the recommendations of the American Institute of Nutrition for laboratory animals (A04 diet from Panlab S.L., Barcelona, Spain). The protocol was approved by the Experimental Animal Committee of the Complutense University of Madrid (Spain). All mice were also treated according to the guidelines of the European Community Council Directives ECC/566/2015. We used female mice due to the fact that males show aggressive and dominant behavior, making it impossible to house them in groups.

2.1. Selection of prematurely aging mice (PAM)

The mice, after one week of acclimatization in the animal room, were marked for their individual follow-ups and were submitted to a T-shaped maze test. This test, which is based on the activity that mice show when they are exposed to the T-maze, was performed once a week for four consecutive weeks to sort out the prematurely aging mice (PAM) (mice that cross the intersection of two arms in > 10 s in all four tests) from the exceptional non-prematurely aging mice (ENPAM) (which cross the intersection of two arms in < 10 s in all four tests) as previously reported (Guayerbas et al., 2002a; Martínez de Toda et al., 2016; Martínez de Toda et al., 2018). We consider these ENPAM because they show a better behavioral and immune system response, together with a longer lifespan, than regular non-prematurely aging mice (R-NPAM) which show an intermediate response in the T-maze and were removed from the study. This test was always performed under red light, between 09:00 and 11:00 h, to minimize circadian variations.

2.2. Experimental design

At the age of 37–38 weeks (adults), 44 ENPAM and 44 PAM were randomly divided into the following groups: one cage for each control group (one for PAM group-Control PAM and another one for ENPAM group-Control ENPAM), each one containing 8 mice; two cages for the ENPAM = 50% and PAM = 50% groups, each one containing 4 ENPAM and 4 PAM; and four cages for the PAM > 50% and ENPAM < 50% groups, each one containing 5 PAM and 2 ENPAM. With respect to PAM < 50%/ENPAM > 50% groups, we had four cages containing in each one 2 PAM and 5 ENPAM. After two months in these living conditions and in order to harmonize the number of animals in all experimental groups, eight animals of the PAM > 50% and ENPAM > 50% groups were randomly selected. All animals corresponding to all experimental groups were then submitted to a battery of behavioral tests in order to evaluate sensorimotor abilities, anxiety-like behaviors and exploratory capacities. Then, peritoneal leukocytes were extracted and several immune functions as well as oxidative stress

parameters were assessed in these same animals. Furthermore, in order to avoid the possible interference that the estrus cycle in adult female mice could produce in the results, we checked the state of this cycle in all mice included in the study the day before beginning the behavioral trials as well as before leukocyte extraction. For this, we did a vaginal scraping of all mice included in the study and we checked the morphology as well as the number of each kind of cell that they showed at this time. The diestrus phase was characterized by the presence of leukocytes, which invade the vaginal epithelium, this kind of cell being the predominant population in this estral phase (Feder, 1981; Freeman, 1988). All animals included in the study were shown to be in the diestrus phase in all tests carried out. Finally, animals were maintained in these living conditions until their natural death in order to analyze their lifespan.

2.3. Behavioral tests

The experiments were performed from 09:00 to 12:00 h in order to avoid possible interferences of circadian rhythms and in accordance with the Spanish legislation on "Protection of Animals Used for Experimental and Other Scientific Purposes" and the European Communities Directives (ECC/566/2015) on this subject. All behavioral tests were performed for five consecutive days. On the first day, animals were subjected to the whole battery of sensorimotor and T-maze tests. On the second and third days, a holeboard test was performed, and, finally, an elevated plus maze was employed on the fourth and fifth days. The sequence follows that of the previous studies carried out by different authors (Baeza et al., 2010; Garrido et al., 2018b; Giménez-Llort et al., 2002), which were based on the stress response that mice can exhibit when they are exposed to this kind of apparatus. The tests were carried out under red light with a white lamp (20 W), and they were started by placing the animals in the area of the apparatus considered most behaviorally neutral so that the mouse was not artificially induced to perform a significant pattern (De Cabo de la Vega et al., 1995). Olfactory trails were removed by cleaning the surface with of the apparatus with 70% ethanol after each mouse.

2.3.1. Sensorimotor abilities

2.3.1.1. Visual placing and hindlimb extensor reflexes. Both kind of reflexes were performed following the protocol previously described (Baeza et al., 2010; Garrido et al., 2018b). For these tests, the mouse was suspended by the tail and lowered toward a solid black surface. Complete extension of the forelimbs as well as complete extension of the hind-limbs was considered a positive response, which was transcribed as a 1. Negative response was considered when complete extension of the forelimbs and hind-limbs was not completed and was transcribed as a 0. The mean response was rated in three trials and calculated as a percentage.

2.3.1.2. Wood rod test. With the objective to evaluate motor coordination, a wood rod test was performed. For this, the mouse was placed in the center of an elevated wooden rod (rod dimensions: 22 cm height, 80 cm length, square section of 2.9 cm, divided in segments of 10 cm) and submitted to one trial of 60 s. Motor coordination was measured by the latency (in seconds) to leave the starting segment and by total number of segments crossed.

2.3.1.3. Tightrope test. The tightrope test, which evaluates the motor coordination, muscular vigor and traction, was performed (Baeza et al., 2010; Garrido et al., 2018b). Mice were hung by their forelimbs in the middle of an elevated horizontal tightrope, which had 40 cm height, 60 cm length and was divided into segments of 10 cm. Motor coordination was evaluated by the latency to leave the starting segment, in seconds, and by the total number of segments crossed. Muscular vigor was determined by the percentage of mice falling off the rope and the latency (seconds) to fall. The percentage of mice finishing the trial and their latency (seconds), were also recorded. Finally, traction was assessed analyzing the different parts of the body that mice used to remain hanging (forelimbs, hind-limbs, and tail). The percentages of mice displaying the low (using forelimbs), medium (using forelimbs and hind-limbs or forelimbs and tail) and maximum traction capacity (using forelimbs, hind-limbs, and tail) were also analyzed.

2.3.2. Exploratory and anxiety-like behavioral tests

2.3.2.1. Holeboard test. Exploratory and anxiety-like behaviors were analyzed following the previously described protocol (Baeza et al., 2010), with slight modifications (Garrido et al., 2018b). The apparatus consists of a box (60 × 60 × 45 cm) with matte-painted metallic walls, divided into 36 squares (10 × 10 cm), bearing four equally spaced holes (3.8 cm of diameter) in the inner zone. We considered the inner zone as four central squares and the external zone as 12 squares besides the walls. Plastic objects were placed in each hole to attain mouse attraction and drive their "goal-directed behavior". The test was performed for 5 min and the parameters recorded for "non-goal directed behavior" were the percentages of total, inner (total number of segments that animal crossed in the inner squares divided by total locomotion) and external locomotion (total number of segments that animal crossed in the external squares divided by total locomotion). These all related to horizontal activity. As vertical activity parameters, the total number of rearing and the time (in seconds) of rearing were recorded. Furthermore, the total number of head-dipping and the time (in seconds) of head-dipping was evaluated as "goal-directed behavior". Finally, self-grooming and freezing behaviors (number and duration, in seconds, of grooming and freezing) were also recorded.

2.3.2.2. T-maze test. The spontaneous horizontal exploratory behavior of the mice was also tested in a T-shaped maze (short arm: 25 × 10 cm; long arm: 65 × 10 cm; walls: 20 cm high) (Baeza et al., 2010; Garrido et al., 2018b). The mouse was placed inside the "vertical" arm of the maze with its head facing the end wall. The performance was evaluated by recording the time (in seconds) elapsed until the animal crossed (four paws criteria) the intersection of the three arms of the maze, as well as the time spent (in seconds) exploring the entire maze.

2.3.2.3. Elevated plus maze. The elevated plus maze, a typical test to evaluate anxiety-like behaviors, was performed following the protocol previously described (Garrido et al., 2018b). The apparatus consists of two opposing open arms (45 × 10 cm) and two enclosed arms (5x10x50 cm) that extend from a central platform (10 × 10 cm), elevated 65 cm above the floor. The mice were individually placed on the central platform facing an enclosed arm and were allowed to freely explore the maze for 5 min. The total number of entries (four paws criteria) in open and closed arms was recorded and their percentages calculated.

2.4. Collection of peritoneal leukocytes

Peritoneal leukocyte suspensions were collected between 08:00 and 12:00 h, with the objective of avoiding the possible interference with circadian variations. These samples, which are minimally invasive, have the advantage that the animals do not have to be sacrificed. Non-anesthetized mice were held by cervical skin, the abdomen was cleansed with 70% ethanol, and 3 ml of sterile Hank's solution, previously warmed to 37 °C, was injected intraperitoneally. After abdominal massage, approximately 80% of the injected volume was recovered. Then, the macrophages and lymphocytes from the peritoneal suspensions identified by their morphology were quantified in Neubauer chambers using optical microscopy (40×). Cellular viability was routinely measured before and after each experiment with the Trypan-blue (Sigma-Aldrich, St. Louis, MO, USA) exclusion test and it was higher than 98% in all cases. The following studies were performed

using unfractionated peritoneal leukocytes to better reproduce *in vivo* immune response and redox state. The peritoneal suspensions were adjusted to a specific number of macrophages, lymphocytes, or total leukocytes, depending on the parameter analyzed, as described in the corresponding section.

2.5. Immune function parameters

2.5.1. Phagocytosis of macrophages

The study of the phagocytic capacity of peritoneal macrophages, adjusted to 5×10^5 macrophages/mL Hank's solution, was carried out as previously described (De la Fuente, 1985). Aliquots of 200 μL of peritoneal suspensions were incubated in MIF culture plates (Sterilin, Teddington, England) for 30 min. Then, 20 μL of latex beads (1.09 mm diluted to 1% in PBS, Sigma, St. Louis, MO), pre-washed with PBS (phosphate buffer saline), were added to the adherent monolayer of cells. After 30 min of incubation, the plates were washed, fixed, and stained with Giemsa stain (Sigma-Aldrich), and the number of latex beads ingested by 100 macrophages (phagocytic index) was determined using an optical microscopy (100×).

2.5.2. Chemotaxis of macrophages and lymphocytes

The chemotaxis assays were performed according to a method previously described (Puerto et al., 2002). This protocol consists of the use of chambers with two compartments separated by a filter (Millipore, Bedford, MA, USA). 300 μl of peritoneal suspensions adjusted to 5×10^5 macrophages/ml Hank's or 5×10^5 lymphocytes/ml Hank's, were deposited in the upper compartment of the chambers and f-met-leu-phe (Sigma, St. Louis, MO, USA) (a positive chemotactic peptide *in vitro*) was placed in the lower compartment. After a 3 h incubation, the filters were fixed and stained, and the chemotaxis index (C.I.) was determined by counting the total number of macrophages or lymphocytes in one third of the lower face of the filters.

2.5.3. Natural killer cytotoxicity

Natural killer cytotoxicity was measured following an enzymatic colorimetric assay (cytotox 96, Promega, Madison, WI, USA) based on the determination of the lactate dehydrogenase (LDH) activity released by the cytolysis of tumor cells, as previously described (Ferrández et al., 1999). Briefly, target cells (YAC-1 cells from a murine lymphoma) were seeded in 96-well U-bottom culture plates (Nunc) at a concentration of 10^4 cells/well in 1640 RPMI without phenol red (PAA). Effector cells (peritoneal leukocytes) were added at a concentration of 10^5 lymphocytes/well, obtaining an effector/target cell rate of 10 to 1. The plates were centrifuged at 250*g* for 5 min to facilitate cell-to-cell contacts and incubated afterwards for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. Then, they were centrifuged again, and lactate dehydrogenase (LDH) enzymatic activity was measured in 50 μL/well supernatant by adding enzyme substrate and recording absorbance at 490 nm. Three kinds of control measurements were performed: target spontaneous release, target maximum release, and effector spontaneous release. To determine the percentage of lysis of target cells, the following equation was used: % lysis = [(*E*-ES-TS)/(M-TS)] x 100, where E is the mean of absorbance values in the presence of effector and target cells, ES the mean of absorbance values of effector cells incubated alone, TS the mean of absorbance values of target cells incubated alone, and M the mean of maximum absorbance values after incubation of target cells with lysis solution.

2.5.4. Lymphoproliferation assay

The lymphoproliferation assay was carried out following a method previously described (Guayerbas et al., 2002b). Lymphoproliferative response to the mitogens concanavalin A (ConA; Sigma-Aldrich) and lipopolysaccharide (LPS, *Escherichia coli*, 055: B5; Sigma-Aldrich) was evaluated. Aliquots of 200 μL of peritoneal leukocyte suspensions adjusted to 10^6 lymphocytes/mL in complete medium containing 1640 RPMI (PAA, Piscataway, NJ, USA) supplemented with gentamicin (10 mg/mL, PAA) and 10% heat-inactivated fetal calf serum (PAA) were dispensed into 96-well plates (Nunc, Roskilde, Denmark). 20 μL/well of complete medium (resting proliferation) or supplemented with ConA (1 μg/mL, Sigma-Aldrich) or with LPS (1 μg/mL, Sigma-Aldrich) (stimulated proliferation) were added. After 48 h of incubation at 37 °C in a sterile and humidified atmosphere of 5% CO_2, 100 μl of culture supernatants were eliminated and 0.5 μCi [3H] thymidine (MP Biomedicals, Santa Ana, CA, USA) was added to each well together with 100 μl of fresh medium. The plates were incubated for an additional 24 h. Finally, the cells were harvested in a semi-automatic harvester (Skatron Instruments, Norway), and thymidine uptake was measured in a beta counter (LKB, Uppsala, Sweden) for 1 min. The lymphoproliferative response was calculated as the number of incorporated counts per minute relativized to those of the resting lymphoproliferation (wells without mitogens) and expressed as the percentage of stimulation.

2.6. Oxidative stress parameters

2.6.1. Catalase activity

Catalase activity was determined following a method previously described (Beers and Sizer, 1952), with slight modifications introduced by our research group (Alvarado et al., 2006a). The enzymatic assay was followed spectrophotometrically for 80 s at 240 nm by the decomposition of H_2O_2 (14 mM in phosphate buffer) into $H_2O + O_2$. The results were expressed as International Units (IU) of enzymatic activity per 10^6 peritoneal leukocytes.

2.6.2. Glutathione reductase activity

The activity of enzyme glutathione reductase was assessed following a method previously described (Massey and Williams, 1965) with some modifications (Alvarado et al., 2006a). This method is based on the oxidation of beta-nicotinamide adenine dinucleotide 2′-phosphate reduced (β-NADPH, 6 mM, Sigma-Aldrich) by this enzyme. The reaction was followed by spectrophotometry at 340 nm for 240 s. The results were expressed as milliunits (mU) of enzymatic activity per 10^6 peritoneal leukocytes.

2.6.3. Glutathione peroxidase activity

Glutathione peroxidase activity was measured using the modified technique previously described (Alvarado et al., 2006a; Lawrence and Burk, 1976). The reaction was followed spectrophotometrically by the decrease of the absorbance at 340 nm for 300 s. The results were expressed as mU of enzymatic activity per 10^6 peritoneal leukocytes.

2.6.4. Glutathione concentrations

Both oxidized (GSSG) and reduced (GSH) forms of glutathione were determined using a fluorimeter, as previously described (Hissin and Hilf, 1976), adapted to 96-well plates, with slight modifications introduced by our research group (Garrido et al., 2017). This procedure is based on the capacity of GSSG to react with o-phthalaldehyde (OPT; Sigma-Aldrich), a fluorescent reagent, at pH 12, and GSH at pH 8, resulting in the formation of a fluorescent compound. Aliquots of 1 mL of the peritoneal suspension (10^6 cells/mL Hank's solution) were centrifuged at 1200 *g* for 10 min at 4 °C. Pelleted cells were re-suspended in phosphate buffer containing EDTA (0.1 M, pH 8; Sigma-Aldrich). Then, samples were sonicated, and after the addition of 5 μL of $HClO_4$ (60%; Sigma-Aldrich), they were centrifuged at 9500 *g* for 10 min at 4 °C. Aliquots of 10 μL of supernatants were dispensed into two 96-well black plates (Nunc), one for each glutathione form. For GSSG measurement, 8 μL of N-ethylmaleimide (NEM, 0.04 M; Sigma-Aldrich) was added to each well to prevent the interference of GSH with the measurement of GSSG, and then incubated at room temperature for 30 min in the dark. Then, 182 μL of NaOH (0.1 N; Panreac) and 20 μL of o-phthalaldehyde (OPT; 1 mg/mL in methanol) were incorporated and the plate was incubated for 15 min under the same conditions. The fluorescence

Table 1
Table 1. Sensorimotor abilities evaluated in C PAM, PAM > 50%, PAM = 50%, PAM > 50%, C ENPAM, ENPAM > 50%, ENPAM = 50% as well as ENPAM < 50%.

	Sensorimotor abilities							
	PAM C (N = 8)	PAM > 50% (N = 8)	PAM = 50% (N = 8)	PAM < 50% (N = 8)	ENPAM C (N = 8)	ENPAM > 50% (N = 8)	ENPAM = 50% (N = 8)	ENPAM < 50% (N = 8)
Weight (g)	40 ± 2	41 ± 1	39 ± 2	40 ± 2	40 ± 1	41 ± 1	41 ± 2	40 ± 2
Visual placing reflex								
% Mice showing this response	100	100	100	100	100	100	100	100
Hindlimb extensor reflex								
% Mice showing this response	100	100	100	100	100	100	100	100
Wood rod test								
Motor coordination								
Latency to leave the starting segment (s)	9 ± 3 ##	6 ± 1 #	5 ± 2	4 ± 1	3 ± 1	4 ± 1 *	6 ± 2	7 ± 1 ##
Total number of crossing segments	4 ± 0	4 ± 1	4 ± 0	4 ± 1	4 ± 0	4 ± 0	4 ± 0	4 ± 0
Equilibrium								
% Mice falling off the rod	0	0	0	0	0	0	0	0
Latency to fall (s)	0	0	0	0	0	0	0	0
% Mice finishing the trial	100	100	100	100	100	100	100	100
Latency to arrive (s)	15 ± 3 #	12 ± 3	8 ± 5	9 ± 3	8 ± 2	9 ± 3	9 ± 3	12 ± 2
Tightrope test								
Motor coordination								
Latency to leave the starting segment (s)	12 ± 6	11 ± 5	11 ± 6	10 ± 3	9 ± 4	12 ± 4	12 ± 6	13 ± 5
Total number of crossing segments	2 ± 1	3 ± 1	2 ± 1	3 ± 1	3 ± 1	2 ± 1	2 ± 1	2 ± 1
Muscular vigor								
% Mice falling off the rope	57 #	33	30	33	25	25	40	48
Latency to fall (s)	9 ± 3 ###	15 ± 0 * ###	18 ± 4 * ##	32 ± 12 **	35 ± 5	17 ± 5 ##	9 ± 2 ###	7 ± 5 ###
% Mice finishing the trial	15 ##	50 *	50 *	50 *	75	63 **	34 ##	30 ## c
Latency to arrive (s)	45 ± 18	41 ± 10	40 ± 1	32 ± 16	29 ± 7	29 ± 11	33 ± 9	40 ± 9
Traction								
Low	85 ###	24 # *	28 # *	17 **	0	20	25	44
Medium	15	38	14	0	15	0	20	44
Maximum	0 ###	38 * ###	38 * ###	83 *** aa bb	85	80 ###	55 # c	22 ** cc d

Each value represents mean ± standard deviation (error) of 8 values corresponding to that number of animals. * p < .05, ** p < 0–01 with respect to C PAM; # p < .05, ## p < .01 and ### p < .001 with respect to C ENPAM; a p < .05 with respect to PAM > 50%; c p < .05 with respect to ENPAM > 50%.

Table 2
Exploratory capacities and anxiety-like behaviors evaluated in C PAM, PAM > 50%, PAM = 50%, PAM > 50%, C ENPAM, ENPAM > 50%, ENPAM = 50% as well as ENPAM < 50%.

	Exploratory capacities and anxiety-like behaviors							
	PAM C (N = 8)	PAM > 50% (N = 8)	PAM = 50% (N = 8)	PAM < 50% (N = 8)	ENPAM C (N = 8)	ENPAM > 50% (N = 8)	ENPAM = 50% (N = 8)	ENPAM < 50% (N = 8)
Holeboard test								
Non-goal directed behavior								
Vertical activity								
Total number of *rearings*	12 ± 5	15 ± 4	25 ± 7	24 ± 5	21 ± 5	20 ± 5	22 ± 8	16 ± 5
Total time of *rearings* (s)	15 ± 3 $^{\#\#\#}$	26 ± 4 *	32 ± 8 **	33 ± 5 ***	37 ± 9	38 ± 8 ***	24 ± 9	21 ± 6
Horizontal activity								
Locomotion								
Inner locomotion	24 ± 3 $^{\#}$	25 ± 7	26 ± 12	39 ± 6 *	41 ± 11	32 ± 9	28 ± 6	21 ± 10
External locomotion	121 ± 27	164 ± 35	159 ± 15	167 ± 32	136 ± 10	154 ± 32	141 ± 57	133 ± 27
Total locomotion	209 ± 34 $^{\#\#\#}$	264 ± 61 $^{\#\#}$	276 ± 43 $^{\#}$	318 ± 22 ***	359 ± 11	282 ± 52 $^{\#}$	250 ± 69 $^{\#\#}$	209 ± 34 $^{\#\#\#}$
Goal directed behavior								
Total number of head-dipping	10 ± 2 $^{\#\#}$	14 ± 4	15 ± 3	20 ± 4 **	23 ± 6	18 ± 2 **	16 ± 2 *	15 ± 3
Total time of head-dipping (s)	17 ± 4 $^{\#\#\#}$	21 ± 3 $^{\#\#\#}$	20 ± 4 $^{\#\#\#}$	37 ± 9 *** aa bb	58 ± 4	29 ± 7 $^{\#\#\#}$ *	29 ± 6 $^{\#\#\#}$ *	16 ± 5 $^{\#\#\#}$ c d
Self-grooming and -freezing behaviors								
Total number of groomings	8 ± 2 $^{\#\#\#}$	3 ± 2 $^{\#}$ *	1 ± 1 ***	0 *** a	0	0 ***	0 ***	3 ± 1 $^{\#}$ * c d
Total time of grooming (s)	12 ± 4 $^{\#\#\#}$	7 ± 3 $^{\#\#\#}$	3 ± 1 $^{\#\#\#}$ ***	0 *** aaa bbb	0	0 ***	0 ***	6 ± 2 $^{\#\#}$ cc dd
Total number of freezings	3 ± 1 $^{\#}$	3 ± 2	0 *	0 *	0	0 *	0 *	1 ± 1
Total time of freezing (s)	5 ± 2 $^{\#\#}$	3 ± 1 $^{\#}$	0 ** a	0 ** a	0	0 *	0 *	2 ± 1
T-maze test								
Horizontal activity								
Time for crossing the intersection of the maze (s)	15 ± 3 $^{\#\#}$	13 ± 4	9 ± 5	8 ± 1 **	8 ± 2	9 ± 2 *	11 ± 3	13 ± 3
Time spent exploring the entire maze (s)	36 ± 4 $^{\#\#\#}$	30 ± 8	23 ± 7 *	22 ± 5 **	20 ± 3	21 ± 4 **	25 ± 7	34 ± 4 $^{\#\#}$ cc
Elevated Plus Maze								
Anxiety-like behaviors								
Total number of entries in open arms	5 ± 2 $^{\#\#\#}$	8 ± 3 $^{\#\#}$	10 ± 2 * $^{\#}$	13 ± 3 **	16 ± 2	12 ± 5	12 ± 3	8 ± 4 $^{\#}$
Total number of entries in closed arms	11 ± 2	12 ± 4	11 ± 3	11 ± 4	9 ± 3	11 ± 3	10 ± 2	11 ± 3
% Time in open arms	17 ± 3 $^{\#\#\#}$	23 ± 2 $^{\#\#\#}$	28 ± 4 * $^{\#\#}$	36 ± 3 *** aa	44 ± 5	34 ± 3 $^{\#}$ ***	30 ± 2 $^{\#}$ ***	23 ± 4 $^{\#\#\#}$ c
% Time in closed arms	62 ± 4 $^{\#\#\#}$	51 ± 4 * $^{\#\#}$	40 ± 4 ** a	34 ± 2 *** aaa	33 ± 3	41 ± 5 ***	45 ± 2 $^{\#\#}$ ***	52 ± 3 $^{\#\#\#}$ cc *

Each value represents mean ± standard deviation (error) of 8 values corresponding to that number of animals. * $p < .05$, ** $p < 0–01$ and *** $p < .001$ with respect to C PAM; $^{\#}$ $p < .05$, $^{\#\#}$ $p < .01$ and $^{\#\#\#}$ $p < .001$ with respect to C ENPAM; a $p < .05$, aa $p < .01$ and aaa $p < .001$ with respect to PAM > 50%; c $p < .05$, cc $p < .01$ with respect to ENPAM > 50%; d $p < .05$ with respect to ENPAM = 50%.

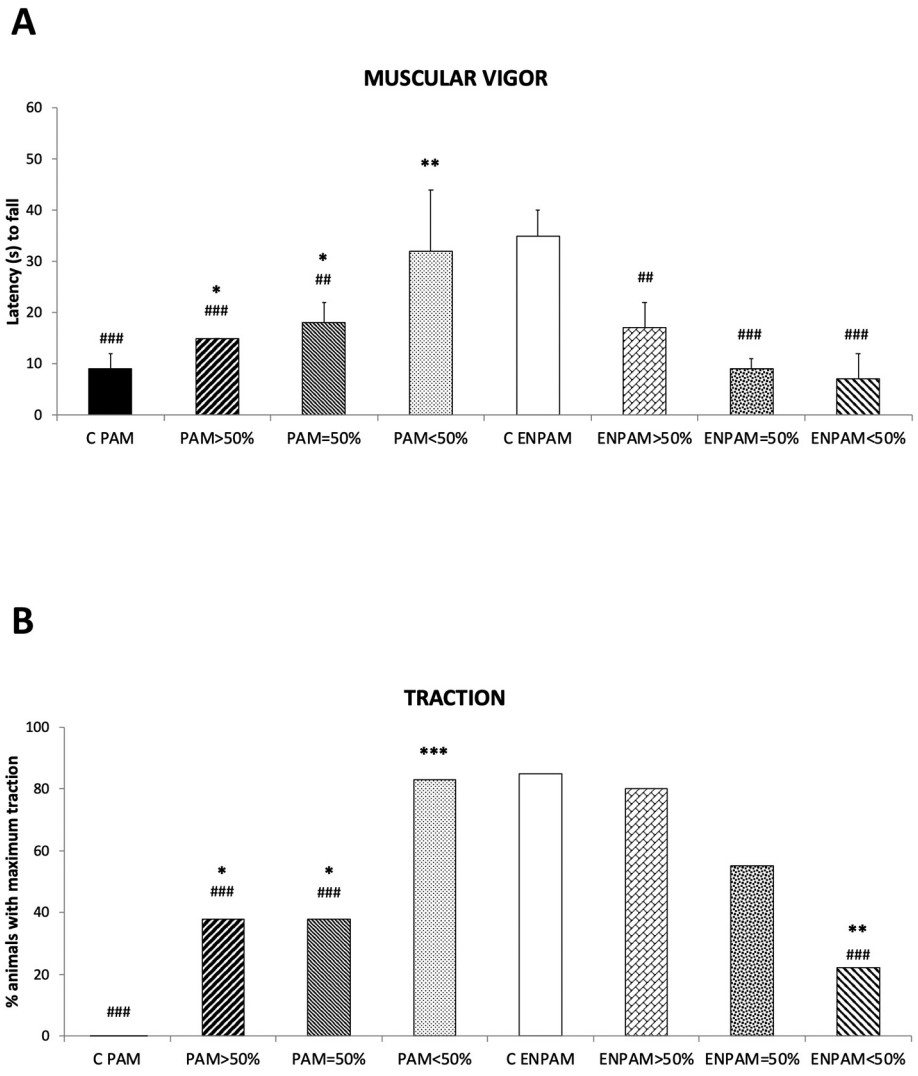

Fig. 1. Sensorimotor abilities. Latency (in seconds) to fall in the tightrope test (A), percentage (%) of animals with maximum traction analyzed in the tightrope test (B) evaluated in C PAM, PAM > 50%, PAM = 50%, PAM < 50%, C ENPAM, ENPAM > 50%, ENPAM = 50% and ENPAM < 50%. Each column represents the mean ± standard deviation of values corresponding to 8 animals. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ with respect to C PAM, $^{\#\#}$ p < .01, $^{\#\#\#}$ p < .001 with respect to C ENPAM.

emitted by each well was measured at 350 nm excitation and 420 emission and the results were expressed as nmol/10^6 peritoneal leukocytes. For measurement of GSH content, 190 μL of phosphate buffer with EDTA and 20 μL of OPT were added to the 10 μL of cell supernatants dispensed in the wells. The plate was incubated for 15 min under the same conditions, and the fluorescence emitted by each well was measured at the same wave-length. The results were expressed as nmol GSH or GSSG per 10^6 peritoneal leukocytes. Also, GSSG/GSH ratios were calculated.

2.6.5. Xanthine oxidase activity

Xanthine oxidase (XO) activity was assayed using a commercial kit (A-22182 Amplex Red Xanthine/Xanthine Oxidase Assay Kit, Molecular Probes, Paisley, UK). In the assay, XO catalyzes the oxidation of purine bases (xanthine/hypoxanthine) to uric acid and superoxide anion. The superoxide spontaneously degrades in the reaction mixture to hydrogen peroxide, which in the presence of horseradish peroxidase (HPR) reacts stoichiometrically with the Amplex Red reagent to generate the red-fluorescent oxidation product resorufin. Aliquots of total leukocytes adjusted to 10^6/mL Hank's solution were lysed in potassium phosphate buffer (0.05 M, pH 7.4) containing EDTA (0.1 M, pH 7.4; Sigma-Aldrich) and dithiothreitol (DTT, 0.5 mM, pH 7.4; Sigma-Aldrich) to prevent reversible Xanthine dehydrogenase (XDH) to XO conversion. In the assay, 50 μL of the lysed solution was incubated with 50 μL working solution of the Amplex Red reagent (100 μM) containing HRP (0.4 U/mL) and xanthine (200 μM). After 30 min of incubation at 37 °C, measurement of fluorescence was performed in a microplate reader (Fluostar Optima, BMG Labtech, Biomedal, Spain) using excitation and emission detection at 530 and 595 nm, respectively. The XO (10 mU/mL) supplied in the kit was used as the standard, and XO activity was measured by comparing the fluorescence of samples with that of standards. The results were expressed as units (U) of enzymatic activity per 10^6 peritoneal leukocytes.

2.7. Mean lifespan

In order to evaluate the possible effects of cohabitation on mean lifespan, all experimental groups were housed in the same conditions until their natural death, which was recorded for the analysis of the lifespan.

2.8. Statistical analysis

The data were expressed as the mean ± standard deviation of the

values. Statistics were performed using SPSS version 21.0 (Chicago, IL, USA). The normality of the samples and the homogeneity of variances were checked by the Kolmogorov-Smirnov and Levene analyses, respectively. Then, multivariate analysis of variance (MANOVA) was used to evaluate differences in prematurely/exceptional non-prematurely aging mice between the > 50%; =50% and < 50% ratios. One-way analysis of variance (ANOVA) was employed to evaluate the differences in all the parameters analyzed in PAM/ENPAM, followed by *post hoc* test analysis or the non-parametric Kruskal-Wallis test. The Turkey test was used for post-hoc comparisons when variances were homogeneous, whereas its counterpart analysis, Games-Howell, was used with unequal variances when they were heterogeneous. For qualitative data, the Chi-square test was used. In the case of mean lifespan, the Kaplan-Maier test was employed. In all statistical studies two-sided $P < .05$ was considered the minimum level of significance.

3. Results

3.1. Behavioral tests

The results corresponding to behavioral tests performed on C PAM, PAM > 50%, PAM = 50%, PAM < 50%, C ENPAM, ENPAM > 50%, ENPAM = 50% and ENPAM < 50% are shown in Table 1 and Table 2 as well as in Fig. 1, Fig. 2 and Fig. 3.

3.1.1. Sensorimotor abilities

The results of behavioral tests are summarized in Table 1 and Fig. 1.A decline in sensorimotor abilities had been associated with aging and has previously been described in animals with chronological and premature aging (Forster et al., 1996; Garrido et al., 2018b; Ingram et al., 1981; Thompson, 2008; Viveros et al., 2007). In fact, C PAM showed an impairment of motor coordination, exhibiting a higher latency to leave the starting segment in the wood rod test ($p < .01$) than C ENPAM. Furthermore, these animals presented a higher latency to arrive in the wood rod test ($p < .05$) with respect to C ENPAM, a result that could indicate an impairment of equilibrium in this group. Also, a lower muscular vigor was observed in C PAM, since these animals exhibited a higher percentage of falling off the rope and lower latency to fall (Fig. 1A) than C ENPAM ($p < .05$ and $p < .001$, respectively). Moreover, these animals showed a lower percentage of finishing the trial in comparison to C ENPAM ($p < .01$) (Fig. 1B). C PAM also presented lower traction, showing a lower maximum (Fig. 1B) ($p < .001$) and higher low traction (p < .001) than C ENPAM. Nevertheless, PAM that cohabited with ENPAM (PAM > 50%, PAM = 50% and PAM < 50%) showed an improvement in muscular vigor and traction, this improvement being higher when the proportion of ENPAM in the cage was increased. In fact, although PAM > 50% and PAM = 50% showed a higher muscular vigor (higher latency to fall) (Fig. 1A) in the tightrope test in comparison to C PAM ($p < .05$), the PAM < 50% group also exhibited a higher value than C PAM ($p < .01$), reaching values similar to those in C ENPAM. Similarly,

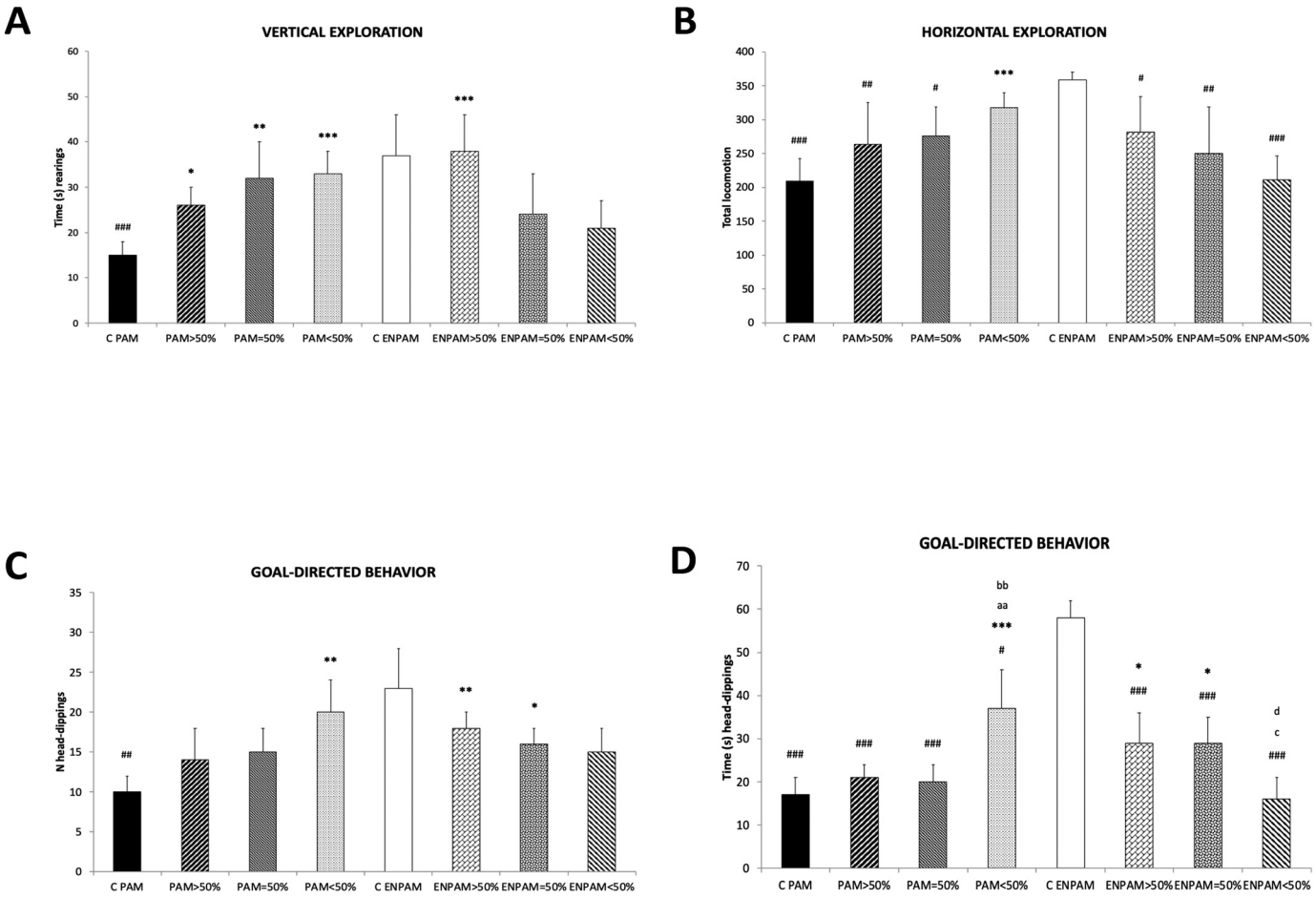

Fig. 2. Exploratory capacities. Time (in seconds) of rearing (A), total locomotion of animals (B), number (N) of head-dipping (C) and time (in seconds) of head-dipping (D) in C PAM, PAM > 50%, PAM = 50%, PAM < 50%, C ENPAM, ENPAM > 50%, ENPAM = 50% and ENPAM < 50% evaluated in the holeboard test. Each column represents the mean ± standard deviation of values corresponding to 8 animals. * p < .05, ** p < .01, *** p < .001 with respect to C PAM, $^{\#}$ p < .05, $^{\#\#}$ p < .01, $^{\#\#\#}$ p < .001 with respect to C ENPAM, aa p < .01 with respect to PAM > 50%, bb p < .01 with respect to PAM = 50%, c p < .05 with respect to ENPAM > 50%, d p < .05 with respect to ENPAM = 50%.

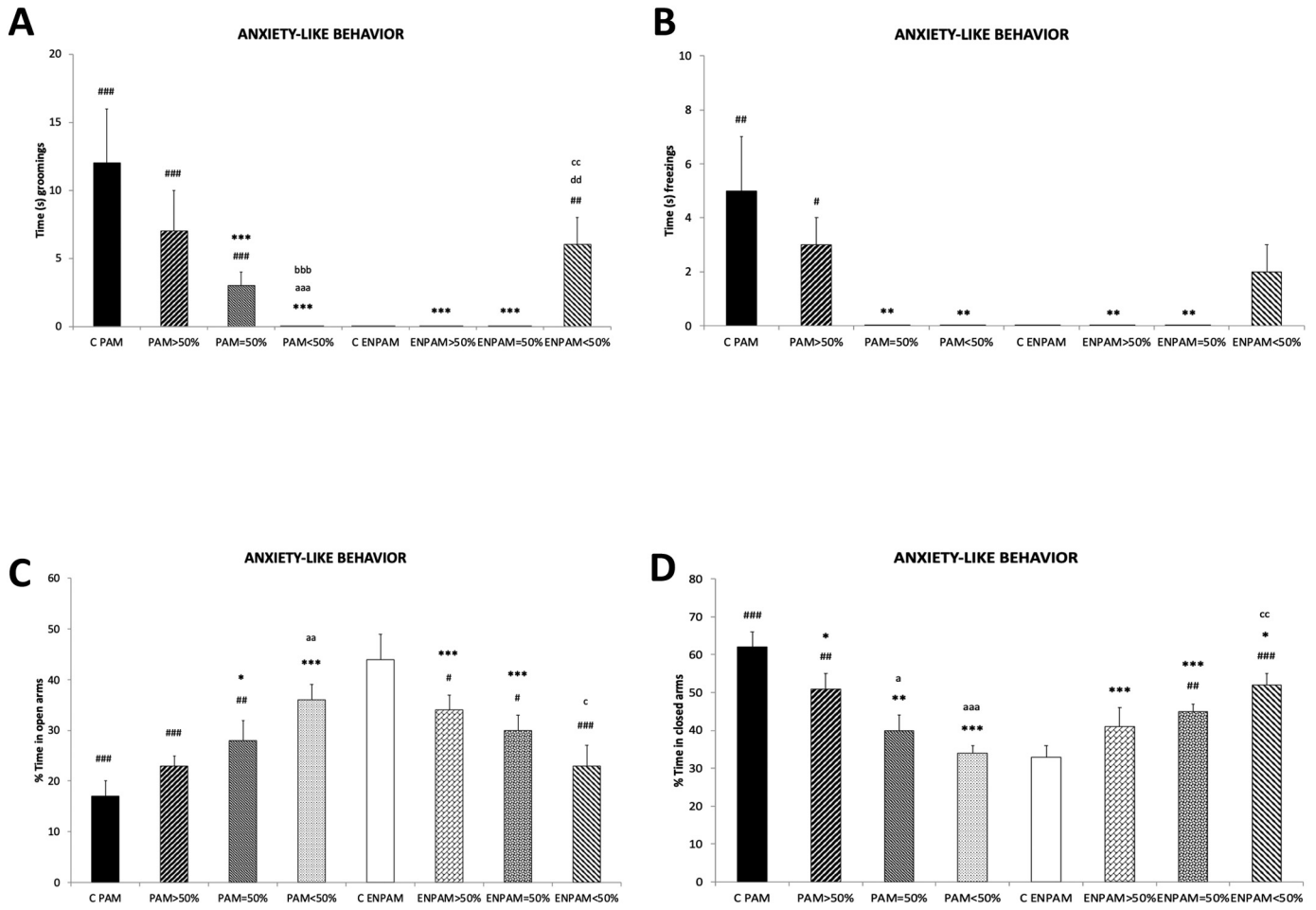

Fig. 3. Anxiety-like behaviors. Time (s) of grooming in holeboard test (A), time (in seconds) of freezing in holeboard test (B), percentage (%) of time in open arms in elevated plus maze (C) percentage (%) of time in closed arms in EPM (D) in C PAM, PAM > 50%, PAM = 50%, PAM < 50%, C ENPAM, ENPAM > 50%, ENPAM = 50% and ENPAM < 50%. Each column represents the mean ± standard deviation of values corresponding to 8 animals. * $p < .05$, ** $p < .01$, *** $p < .001$ with respect to C PAM, $^{\#}$ $p < .05$, $^{\#\#}$ $p < .01$, $^{\#\#\#}$ $p < .001$ with respect to C ENPAM, a $p < .05$, aa $p < .01$, aaa $p < .001$ with respect to PAM > 50%, bbb $p < .001$ with respect to PAM = 50%, c $p < .05$, cc $p < .01$ with respect to ENPAM > 50%, dd $p < .01$ with respect to ENPAM = 50%.

PAM > 50%, PAM = 50% and PAM < 50% exhibited a higher percentage of finishing the tightrope test in comparison to C PAM ($p < .05$), reaching the values of those in the C ENPAM group. With respect to traction, PAM > 50%, PAM = 50% and PAM < 50% showed a lower low traction ($p < .05$) and a higher maximum traction ($p < .05$) than C PAM, PAM < 50% reaching similar values to those of C ENPAM (Fig. 1B). Thus, when PAM cohabited with ENPAM their sensorimotor abilities were improved.

However, ENPAM after cohabiting with PAM for two months exhibited several sensorimotor ability impairments, although these were more noticeable in the ENPAM < 50% group. In fact, ENPAM > 50%, ENPAM = 50% and ENPAM < 50% showed a lower muscular vigor (all these groups exhibited lower latency in falling in the tightrope test than C ENPAM; $p < .01$ and $p < .001$, respectively) (Fig. 1A). In the case of ENPAM = 50% and ENPAM < 50% a lower percentage of mice finished the trial ($p < .01$) in comparison to C ENPAM. Moreover, ENPAM < 50% exhibited a lower motor coordination, showing higher latency to leave the starting segment in the wood rod test ($p < .01$) and lower maximum traction ($p < .05$) than C ENPAM.

3.1.2. Exploratory and anxiety-like behaviors

Regarding exploratory and anxiety-like behaviors, the results are summarized in Table 2 and in Figs. 2 and 3. With respect to exploratory capacity, as shown in Fig. 2A, C PAM showed a lower time of rearing ($p < .001$), a parameter indicative of vertical activity, as well as a lower inner ($p < .05$) (Table 2) and total ($p < .001$) (Fig. 2B) locomotion than C ENPAM, analyzed in the holeboard test. Furthermore, these PAM exhibited higher times in crossing the intersection of the T-maze ($p < .01$) together with a higher time spent exploring the entire maze ($p < .001$) than C ENPAM (Table 2). C PAM also showed a lower frequency ($p < .01$) and time ($p < .001$) of head-dipping in the holeboard test than C ENPAM (Fig. 2C and D), parameters related to goal-directed behavior.

Nevertheless, PAM after cohabiting with ENPAM for two months improved some of these parameters related to vertical and horizontal exploration. In fact, PAM > 50%, PAM = 50% and PAM < 50% exhibited higher times of rearing than C PAM ($p < .05$, $p < .01$ and $p < .001$, respectively), reaching similar values to those in C ENPAM (Fig. 2A). PAM = 50% also spent less time to explore the entire T-maze than C PAM ($p < .05$), reaching similar values to those in C ENPAM. Furthermore, PAM < 50% had higher inner ($p < .05$) and total ($p < .001$) locomotion (Fig. 2B) evaluated in the holeboard test as well as spending less time to cross the intersection and explore the entire T-maze in comparison to C PAM ($p < .01$), reaching values similar to the C ENPAM group. With respect to goal-directed behavior, PAM < 50% had a higher number of head-dipping (Fig. 2C) as well as spending more time (Fig. 2D) than the C PAM ($p < .01$ and $p < .001$, respectively), reaching similar values in the case of the number of head-dipping to those in C ENPAM. Moreover, PAM < 50% also presented higher times of head-dipping than PAM > 50% and PAM = 50% ($p < .01$)

(Fig. 2D).

With respect to the ENPAM, after cohabitation with PAM for two months, they exhibited an impairment in exploratory capacities. In fact, ENPAM > 50%, ENPAM = 50% and ENPAM < 50% showed lower horizontal exploration (Fig. 2B), evaluated as total locomotion in the holeboard test, than C ENPAM ($p < .05$, $p < .01$ and $p < .001$, respectively), and reaching similar values to those in C PAM. In the case of goal-directed behavior (Fig. 2D), all these experimental groups presented lower times of head-dipping in comparison to C ENPAM ($p < 0{,}001$), although only ENPAM < 50% reached similar values to those in C PAM. Indeed, in this parameter, ENPAM < 50% exhibited lower times than ENPAM > 50% and ENPAM = 50% ($p < .05$). Furthermore, these animals exhibited higher times exploring the entire T-maze than C ENPAM ($p < .01$) and than ENPAM > 50% ($p < .01$), reaching similar values to those in C PAM.

In the case of anxiety-like behaviors, C PAM exhibited a higher total number (Table 2) and time (Fig. 3A) of grooming ($p < .001$) together with a higher total number (Table 2) and time (Fig. 3B) of freezing ($p < .05$ and $p < .01$, respectively), evaluated in the holeboard test, with respect to the C ENPAM group. In the elevated plus maze the C PAM group showed a lower number (Table 2) and percentage (Fig. 3C) of entries in open arms ($p < .001$) as well as a higher percentage of time in closed arms ($p < .001$) (Fig. 3D) than C ENPAM.

In the case of PAM that cohabited with ENPAM, all groups improved these responses, which were better when the number of ENPAM in the cage was increased. Thus, PAM > 50% showed a lower percentage of time in closed arms and total number of grooming than C PAM ($p < .05$), although they did not show similar values to those in the C ENPAM group. Similarly, in the case of PAM = 50%, these animals displayed a higher percentage of time in open arms (Fig. 3C) and a lower percentage of time in closed arms ($p < .05$) (Fig. 3D) in comparison to the C PAM. Indeed, PAM > 50% and PAM = 50% groups exhibited a lower total number and time (Fig. 3A) of grooming ($p < .001$) and freezing ($p < .01$ and $p < .05$, respectively) (Fig. 3B), behaviors evaluated in the holeboard test, than C PAM, reaching similar values in the number and time of freezing as the C ENPAM.

By contrast, ENPAM after living with PAM for two months impaired their anxiety-like response, this being worse when the number of PAM in the cage was increased. In fact, ENPAM > 50%, ENPAM = 50% and ENPAM < 50% groups showed a lower percentage of time in open arms ($p < .05$, $p < .05$ and $p < .001$, respectively) together with a higher percentage of time in closed arms in the case of ENPAM = 50% and EMPAM < 50% mice ($p < .01$ and $p < .001$, respectively) in comparison to C ENPAM (Fig. 3C and D). Furthermore, ENPAM < 50% also presented a higher time of grooming ($p < .01$) (Fig. 3A) in comparison to C ENPAM. Furthermore, ENPAM < 50% had a lower percentage of time in open arms ($p < .05$) together with a higher percentage of time in closed arms ($p < .05$) and higher total number ($p < .05$) and time of grooming ($p < .01$) than ENPAM > 50%. In fact, in this latter parameter, ENPAM < 50% also had a higher total number ($p < .05$) and time of grooming ($p < .01$) than ENPAM = 50%.

3.2. Immune functions parameters

The results corresponding to immune function parameters are illustrated in Fig. 4. Regarding innate immunity functions, which are decreased with aging (De la Fuente and Bauer, 2016; Gruver et al., 2007; Hazeldine and Lord, 2015; Salminen et al., 2008; Weyand and Goronzy, 2016), peritoneal leukocytes from C PAM showed lower phagocytic capacity ($p < .001$) (Fig. 4A) and chemotaxis of both macrophages ($p < .001$) (Fig. 4B) and lymphocytes ($p < .001$) (Fig. 4C) than C ENPAM. Also, as shown in Fig. 4D, these leukocytes had lower cytotoxic NK activity ($p < .001$) in comparison to C ENPAM. With respect to lymphoproliferative response to LPS (Fig. 4E) and ConA (Fig. 4F), immune functions of acquired immunity, the C PAM group showed lower values of proliferative stimulation ($p < .001$) than C ENPAM.

However, PAM after two months living with ENPAM improved these functions, although this beneficial effect was more significant in PAM = 50% and PAM < 50%. In this context, PAM = 50% had a higher cytotoxic NK activity ($p < .01$) (Fig. 4D) than C PAM, reaching similar values to those observed in C ENPAM. In fact, this improvement observed in PAM = 50% was also significantly different from that observed in PAM < 50%, being higher in the former ($p < .01$). Furthermore, these animals also presented a higher lymphoproliferative response to both mitogens (LPS and ConA) ($p < .05$) (Fig. 4E and F) in comparison to C PAM, although in this case the values did not reach those exhibited by leukocytes from C ENPAM. In the case of PAM < 50%, peritoneal leukocytes from this group presented higher values of all the immune functions analyzed than the other PAM groups. In fact, PAM < 50% exhibited higher phagocytic capacity ($p < .001$) (Fig. 4A), macrophage ($p < .001$) (Fig. 4B) and lymphocyte ($p < .001$) (Fig. 4C) chemotaxis, NK cytotoxicity ($p < .001$) (Fig. 4D) as well as higher percentage of stimulation of lymphoproliferation in response to LPS ($p < .05$) (Fig. 4E) and ConA ($p < .05$) (Fig. 4F) than C PAM, although the values did not reach those of C ENPAM in the case of lymphoproliferative response to LPS and ConA. Nevertheless, PAM < 50% also showed higher phagocytic capacity ($p < .001$), chemotaxis of macrophages ($p < .001$) and lymphocytes ($p < .05$) as well as NK cytotoxicity ($p < .001$) than PAM > 50%.

By contrast, ENPAM after two months of cohabitation with PAM showed a general impairment of immune function parameters. In fact, peritoneal leukocytes from ENPAM > 50%, ENPAM = 50% and ENPAM < 50% had lower phagocytic capacity ($p < .001$) (Fig. 4A), macrophage and lymphocyte chemotaxis ($p < .001$) (Fig. 4B and C) as well as a lower percentage of stimulation to lymphoproliferative response to both mitogens ($p < .001$) (Fig. 4E and F) in comparison to values observed in C ENPAM and reaching similar values to those in the C PAM group. However, in the case of NK cytotoxicity (Fig. 4D), only peritoneal leukocytes from ENPAM < 50% were lower than C ENPAM, this value also being lower than in ENPAM > 50% ($p < .001$) and ENPAM = 50% ($p < .05$).

3.3. Oxidative stress parameters

The results corresponding to oxidative stress parameters are shown in Fig. 5. With the objective to analyze the redox state of peritoneal leukocytes from C PAM, PAM > 50%, PAM = 50%, PAM < 50% as well as ENPAM > 50%, ENPAM = 50%, ENPAM < 50% and C ENPAM, CAT activity and GSH amounts, as antioxidant defenses, and XO activity and GSSG amounts, as oxidant compounds, were assessed. GSSG/GSH ratios were also calculated.

With regards to antioxidant defenses, which are shown in Fig. 5A and B, peritoneal leukocytes from C PAM and PAM > 50% had lower CAT activities ($p < .001$) as well as GSH amounts ($p < .001$) in comparison to C ENPAM. However, PAM = 50% presented higher CAT activity ($p < .05$) and GSH amounts ($p < .05$) than C PAM, although the values did not reach those of C ENPAM. Nevertheless, PAM < 50% showed higher CAT activities ($p < .01$) and GSH amounts ($p < .001$) than C ENPAM, reaching values shown by C ENPAM. In fact, these animals presented higher CAT activities ($p < .01$) as well as GSH amounts ($p < .01$) than PAM > 50%. In contrast, peritoneal leukocytes from ENPAM > 50% had lower CAT activities ($p < .001$,) than C ENPAM. Similarly, ENPAM = 50% and ENPAM < 50% exhibited lower CAT activities ($p < .01$), although these groups also presented lower GSH amounts ($p < .001$) than C ENPAM. In fact, in the case of GSH amounts, ENPAM = 50% and ENPAM < 50% also had lower values ($p < .01$) in comparison to ENPAM > 50%, reaching in the case of ENPAM < 50% similar values to those by C PAM.

With respect to oxidant compounds, peritoneal leukocytes from C PAM had higher XO activities ($p < .001$) (Fig. 5.C) and GSSG/GSH ratios ($p < .001$) (Fig. 5D) than C ENPAM. However, in the case of

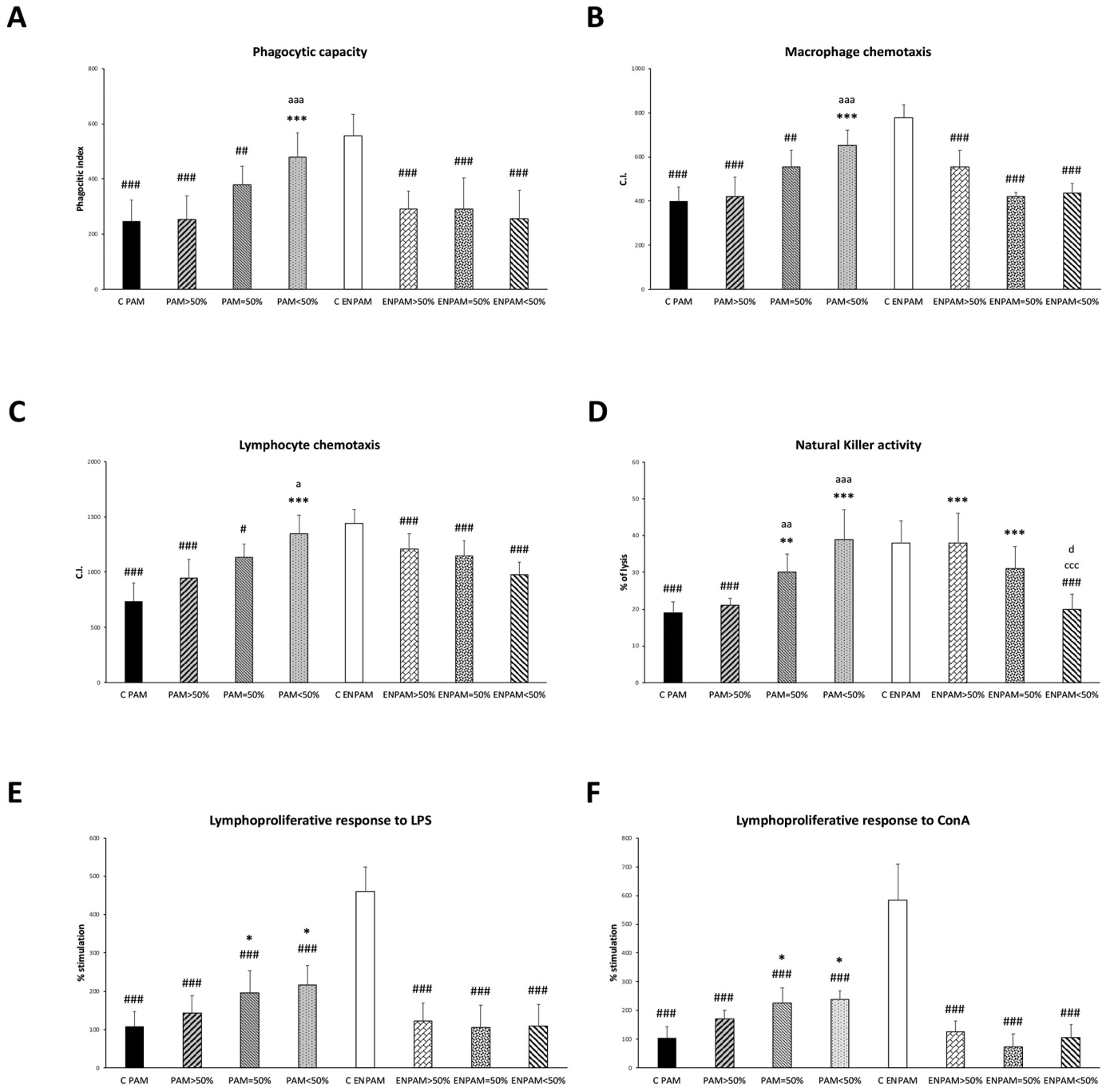

Fig. 4. Immune function parameters. Phagocytic index evaluated in macrophages (PI) (A), chemotaxis index (CI) evaluated in macrophages (B), chemotaxis index (CI) evaluated in lymphocytes (C) percentage (%) of lysis (D), percentage (%) of stimulation in lymphoproliferation in response to LPS (E), percentage (%) of stimulation in response to ConA (F) evaluated in peritoneal leukocytes from C PAM, PAM > 50%, PAM = 50%, PAM < 50%, C ENPAM, ENPAM > 50%, ENPAM = 50% and ENPAM < 50% groups. Each column represents the mean ± standard deviation of values corresponding to 8 animals. * $p < .05$, ** $p < .01$, *** $p < .001$ with respect to C PAM, $^{\#}$ $p < .05$, $^{\#\#}$ $p < .01$, $^{\#\#\#}$ $p < .001$ with respect to C ENPAM, a $p < .05$, aa $p < .01$, aaa $p < .001$ with respect to PAM > 50%, ccc $p < .001$ with respect to ENPAM > 50%, d $p < .05$ with respect to ENPAM = 50%.

GSSG amounts, no differences were observed between all groups (4 ± 1 nmol GSSG/10^6 peritoneal leukocytes). However, PAM > 50%, PAM = 50% and PAM < 50% showed lower XO activities ($p < .001$) (Fig. 5C) in comparison to C PAM, reaching similar values to those of C ENPAM. In the case of GSSG/GSH ratios (Fig. 5D), PAM > 50%, PAM = 50% and PAM < 50% exhibited lower values ($p < .001$) than C PAM, although only PAM = 50% and PAM < 50% had lower values ($p < .001$) than PAM > 50%. In fact, PAM < 50% presented lower GSSG/GSH ratios ($p < .05$) than PAM = 50%. By contrast, although only ENPAM < 50% had higher XO activity values in comparison to C ENPAM, in the case of GSSG/GSH ratios, ENPAM > 50%, ENPAM = 50% and ENPAM < 50% showed higher values than C ENPAM, this being higher, when the proportion of ENPAM in the cage was increased, although neither group reached similar values to those of C PAM. In fact, ENPAM = 50% and ENPAM < 50% had higher ratios ($p < .001$) than ENPAM > 50%.

3.4. Mean lifespan

As shown in Fig. 6, PAM < 50% showed higher mean lifespan than

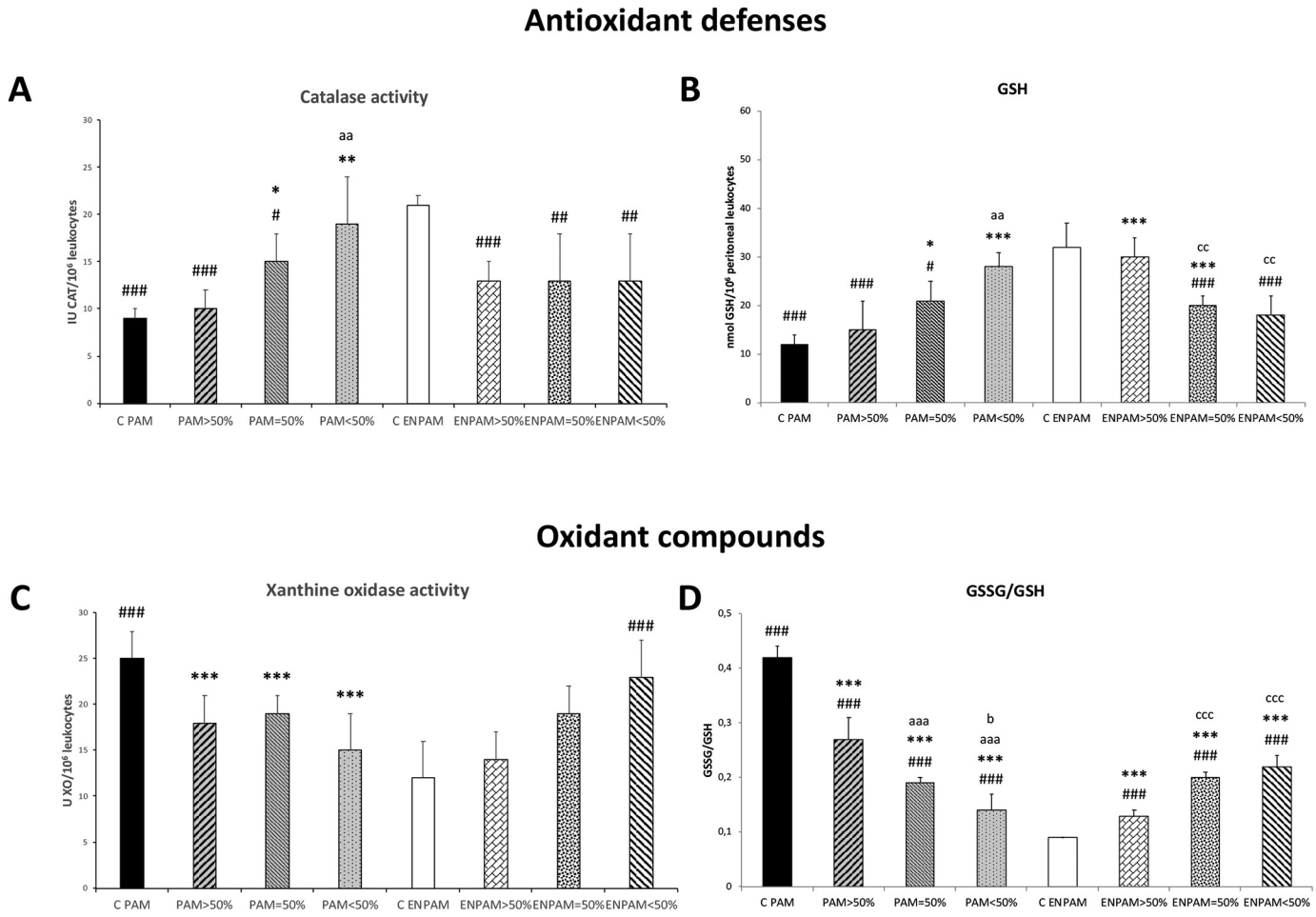

Fig. 5. Oxidative stress parameters. Catalase (CAT) activity (IU CAT/10^6 peritoneal leukocytes) (A), reduced glutathione (GSH) amounts (nmol GSH/10^6 peritoneal leukocytes) (B), Xanthine Oxidase (XO) activity (U XO/10^6 peritoneal leukocytes) (C) and GSSG/GSH ratio (D), evaluated in peritoneal leukocytes from C PAM, PAM > 50%, PAM = 50%, PAM < 50%, C ENPAM, ENPAM > 50%, ENPAM = 50% and ENPAM < 50% groups. Each column represents the mean ± standard deviation of values corresponding to 8 animals. * p < .05, ** p < .01, *** p < .001 with respect to C PAM, $^{\#}$ p < .05, $^{\#\#}$ p < .01, $^{\#\#\#}$ p < .001 with respect to C ENPAM, aa p < .01, aaa p < .001 with respect to PAM > 50%, cc p < .01, ccc p < .001 with respect to ENPAM > 50%, d p < .05 with respect to ENPAM = 50%.

C PAM (p < .01) as well as PAM > 50% (p < .05), reaching similar values to those in C ENPAM. However, no differences were observed in the case of the ENPAM groups.

4. Discussion

This work is the first study in which the effects of cohabitation of different ratios of PAM/ENPAM on behavioral responses, peritoneal immune cell functions and redox state, as well as on lifespan, have been analyzed. The results have shown that for PAM, the cohabitation with ENPAM was very positive when the number of ENPAM in the cage was higher than 50% of the total number of animals (PAM < 50% group). These PAM exhibited a significant improvement in all the aspects studied, resulting in a longer mean lifespan. Furthermore, the beneficial effects observed in the PAM group after two months of cohabitation were gradual and dependent on the number of ENPAM, being more patent when the number of ENPAM in the cage was higher. However, these social cohabitations showed negative consequences in ENPAM, with behavioral and immune impairments, although these did not affect their longevity.

A common feature of aging in the nervous system is the impairment of its functions such as sensorimotor capacities and cognitive abilities, among others. These alterations appear to be related to a lower quality of life (Brenes et al., 2008; Buchman et al., 2009; Forster et al., 1996; Krampe, 2002). Thus, we performed a battery of behavioral tests, analyzing sensorimotor and exploratory capacities and anxiety-like behaviors in PAM and ENPAM after their two month cohabitation. The results showed that C-PAM had lower motor coordination, muscular vigor, and exploratory capacities, as well as higher anxiety-like behaviors, in comparison with ENPAM, as we have previously described in these animals (Guayerbas et al., 2002a; Pérez-Álvarez et al., 2005; Viveros et al., 2007). These characteristics have also been observed in chronologically old mice (Boguszewski and Zagrodzka, 2002; Buchman et al., 2009; Forster et al., 1996; Ingram et al., 1981; Lamberty and Gower, 1992; Thompson, 2008). However, PAM that cohabited with ENPAM, exhibited an improvement of muscular vigor and traction together with higher vertical and horizontal exploration and goal-directed behavior as well as lower anxiety-like behaviors than C-PAM. These beneficial effects on behavioral responses have also been observed in chronologically old mice after cohabiting two months in a predominantly adult environment (Garrido et al., 2018a), and with other environmental strategies that suppose a social and physically active life. In fact, chronologically old mice living in an enriched environment presented higher sensorimotor abilities, exploratory capacities as well as lower anxiety-like behaviors than their corresponding old controls (Arranz et al., 2010b; Benaroya-Milshtein et al., 2004). Also, in senescence-accelerated mice prone 8 (SAMP8), the enriched environment produced similar effects (Griñán-Ferré et al., 2016;

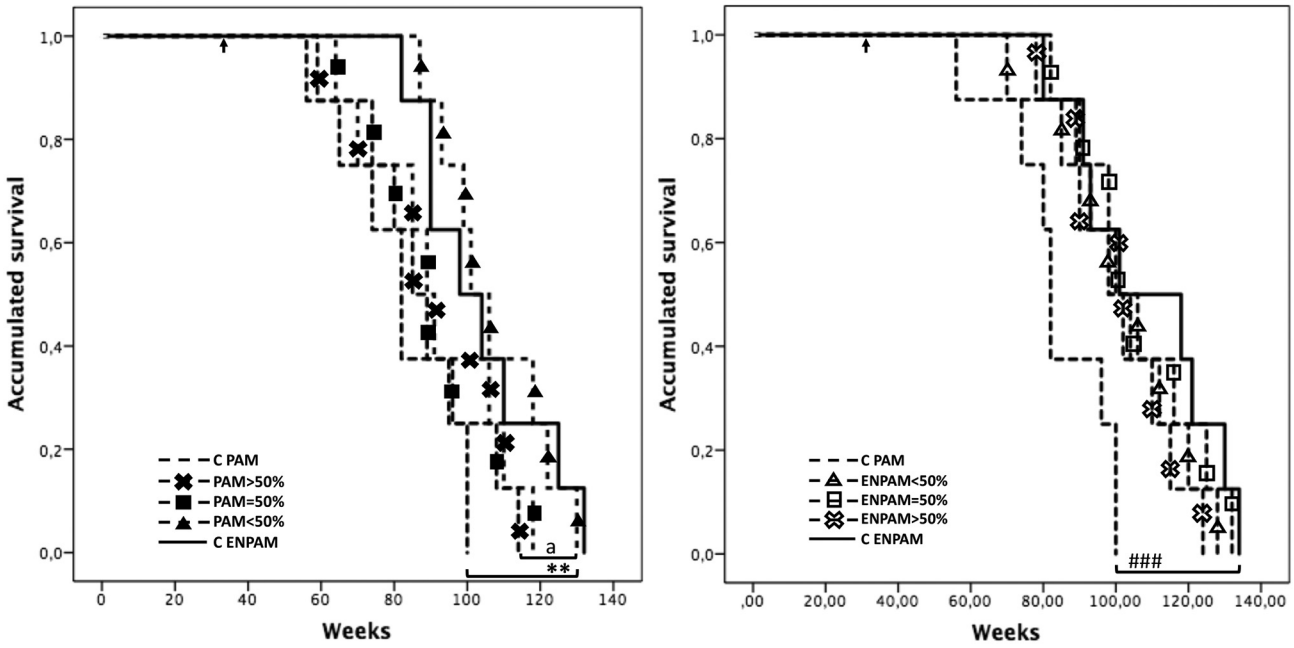

Fig. 6. Mean lifespan. Mortality records of C PAM; PAM > 50%, PAM = 50%, PAM < 50%, C ENPAM, ENPAM > 50%, ENPAM = 50% and ENPAM < 50% groups (N = 8) until the natural death of the animals. ** p < .01 with respect to C PAM; ### p < .001 with respect to C ENPAM; a p < .05 with respect to PAM > 50%.

Griñan-Ferré et al., 2016). It is relevant that the improvement in PAM depends on the number of ENPAM that cohabit in the same cage, the positive effects being higher when this proportion increased. Thus, in PAM < 50% the highest sensorimotor abilities and lowest anxiety-like behaviors were observed. These PAM reached, in general, similar values in the parameters studied to those exhibited by C-ENPAM.

With respect to the immune system, its components undergo striking re-structuring in the aging process, leading to changes that may include enhanced as well as diminished functions. This immunosenescence results in the impairment of both innate and adaptive immunity (De la Fuente and Bauer, 2016; Gruver et al., 2007; Hazeldine and Lord, 2015; Salminen et al., 2008; Weyand and Goronzy, 2016). In fact, several immune functions related to innate immunity, such as phagocytic capacity, lymphocyte and macrophage chemotaxis as well as NK cytotoxic activity and also related to acquired immunity, like the lymphoproliferative response to the mitogens LPS and ConA decrease with aging (Arranz et al., 2010a; De la Fuente et al., 1998; Fulop et al., 2004; Martínez de Toda et al., 2016; Powden et al., 2004; Solana et al., 2012). These impairments have been previously described in PAM (Alvarado et al., 2006b; Guayerbas and De la Fuente, 2003; Guayerbas et al., 2002a, 2002b; Martínez de Toda et al., 2016) and also observed in the C-PAM group of the present study. Nevertheless, higher values of all these immune functions have been observed in PAM after cohabiting with ENPAM for two months, results that support the idea that this social context improves the immune response. In this sense, a higher NK activity in peripheral blood has been observed in older adults with a positive social context (Lutgendorf et al., 2005). Moreover, mice after living in a positive social environment exhibited faster wound healing (Detillion et al., 2004; Glasper and DeVries, 2005), a process closely linked to good innate immunity. Chronologically old mice, after being housed in an enriched social environment, exhibited higher NK activity in spleen in comparison to control animals (Benaroya-Milshtein et al., 2004). Peritoneal leukocytes from old mice living in an enriched environment also showed an improvement of immune functions (Arranz et al., 2010b). In addition, this improvement in immune functions has also been observed in peritoneal leukocytes from chronologically old mice that have cohabited in a predominantly adult environment (Garrido et al., 2018a), and in spleen and thymus leukocytes from PAM after living in a predominantly ENPAM environment (Garrido et al., 2019a, 2019b). However, the number of ENPAM in this cohabitation seems to be important, because the improvement observed in PAM depended on the number of ENPAM in the cage. In fact, it was necessary for the number of ENPAM in this cohabitation to be equal or higher than 50% of PAM in order to improve the immune functions in PAM. While PAM > 50% did not show any improvement, PAM < 50% exhibited higher values in all immune functions analyzed in this study, results that seem to indicate that the beneficial effects of this cohabitation are gradual and dependent on the number of ENPAM. Nevertheless, in the particular case of NK cytotoxic activity and the lymphoproliferative response to mitogens, both PAM = 50% and PAM < 50% exhibited a similar improvement, reaching in the specific case of NK activity, the values observed in C ENPAM. One possible reason for this result observed in NK activity could be the use of unfractionated peritoneal leukocytes. In fact, this activity is produced by different immune cells, such as phagocytes, lymphocytes $CD8^+$ and innate lymphoid cells (ILCs), which include classical cytotoxic NK cells (termed ILC1s), among other cells (Simoni and Newell, 2018; Spits et al., 2013; Trabanelli et al., 2018). Thus, the greatest improvement in this immune function could be due the cumulative beneficial effects of cohabitation on several immune cells with this capacity, although future experiments are needed in order to corroborate this hypothesis. Moreover, cytotoxic activity has been considered as one of the most sensitive immune functions of neuroimmunoendocrine system deregulation (Mocchegiani and Malavolta, 2004), being recently proposed as a marker of biological age and predictor of longevity (Martínez de Toda et al., 2016). Thus, a higher cytotoxic activity observed in the PAM group after cohabitation with ENPAM for two months could be indicative of a recovery of the immune homeostasis in these animals. In the case of lymphoproliferative response, this function is very affected during aging (Castle, 2000; Miller, 1996; Pawelec et al., 2002), but the appropriate modulation of this activity has been proposed as a possible contributor to longevity (Martínez De Toda et al., 2017). Thus, this social environment seems capable of diminishing the premature immunosenescence shown by PAM, exerting beneficial effects when the proportion of PAM/ENPAM is, as least, 50%. These improvements were greater when the number of ENPAM in the cage increased, showing that the beneficial effects of this cohabitation are dependent on the number of ENPAM.

Aging is associated with the establishment of oxidative stress, which is due to an imbalance between oxidant compounds and antioxidant defenses, in favor of the former (De la Fuente and Miquel, 2009; Dröge, 2003; Vida et al., 2017). In fact, previous reports have described this redox imbalance in different tissues and in immune cells from chronologically old mice (Liu et al., 2013; Vida et al., 2017) as well as from PAM (Alvarado et al., 2006b; Garrido et al., 2019a, 2019b; Martínez de Toda et al., 2020). In agreement with this, the C PAM of this study presented higher oxidant compounds together with lower antioxidant defenses than C ENPAM, exhibiting a clear oxidative stress establishment. However, PAM after cohabiting with ENPAM for two months (PAM = 50% and PAM < 50%) showed higher antioxidant defenses (CAT activities and GSH amounts) than C PAM, reaching similar values to those in C ENPAM. A high CAT activity, such as that shown by transgenic mice for this enzyme, has been related to a delay in aging and an extended lifespan (Cutler, 2005; Enns et al., 2008). Furthermore, these PAM that cohabited with ENPAM, exhibited lower oxidant compounds (XO activities) than C-PAM, results that seem to indicate that this environmental enrichment is able to diminish the oxidative stress establishment, previously observed in PAM. In fact, GSSG/GSH ratios, a redox marker (Kard'ar, 2016), were lower in these experimental groups than in C PAM. A similar improvement in redox state has been observed in peritoneal leukocytes from chronologically old mice cohabiting in a predominantly adult environment (Garrido et al., 2018a), in spleen and thymus leukocytes from PAM after cohabiting in a predominantly ENPAM environment (Garrido et al., 2019a, 2019b), and in peritoneal leukocytes from TH-HZ mice cohabiting in a predominantly wild type environment (Garrido et al., 2017). Moreover, this redox improvement was also detected in peritoneal immune cells from chronologically old mice living in an enriched environment (Arranz et al., 2010b). Thus, these results seem to indicate that the cohabitation with a minimum of 50% of ENPAM avoids the chronic oxidative stress of PAM, with an increase in the antioxidant defenses. In the case of the oxidant compounds (XO activities and GSSG/GSH ratios), the improvement was observed even in PAM > 50%. Thus, the beneficial effects of cohabitation are higher in oxidant compounds than in antioxidants. Since oxidative stress is closely linked to immunosenescence (De la Fuente and Bauer, 2016; Hazeldine and Lord, 2015; Salminen et al., 2008), the improvement observed in the redox balance from PAM after cohabiting with ENPAM for two months, could be one reason for the avoidance of the immunosenescence shown by PAM.

In contrast, ENPAM that cohabited with PAM exhibited an impairment of the behavioral responses evaluated in this work, showing lower sensorimotor abilities, such as muscular vigor and traction, horizontal exploration and goal-directed behavior together with higher anxiety-like behaviors. Moreover, these ENPAM also showed premature immunosenescence and oxidative stress in their peritoneal leukocytes. Similar results have been described in adult mice after cohabiting two months with chronologically old mice (Garrido et al., 2018a) and in adult controls after living with adult TH-HZ mice (Garrido et al., 2017). Although we do not know the origin of these impairments, these could be due to the psychological stress produced by the cohabitation with PAM, since this stress alters behavioral responses and immune functions (Cohen et al., 2007; Glaser, 2005; Kennedy, 2016). In fact, when healthy mice lived with sick partners, they showed behavioral abnormalities as well as immunosuppression, which were consequences of the psychological stress that the healthy animals suffered (Morgulis et al., 2004; Palermo-Neto and Alves, 2014). Since this stress has been related to an increased oxidative stress (Irie et al., 2001; Moller et al., 1996), and immunosenescence (Elwenspoek et al., 2017; Prather et al., 2018), two closely linked processes (De la Fuente and Bauer, 2016; Garrido et al., 2019b; Hazeldine and Lord, 2015; Salminen et al., 2008), the redox imbalance observed in these ENPAM after the cohabitation for two months could be responsible for the inadequate immune function in their peritoneal leukocytes. Nevertheless, in our study this impairment was more evident in ENPAM that cohabited in a predominantly PAM environment (ENPAM < 50%), although in several parameters we observed a cumulative effect dependent on the PAM/ENPAM ratio. In agreement with this and as commented earlier, the negative effects on behavior and immune function are more patent in healthy animals when these animals live with an increased number of sick partners (Alves and Palermo-Neto, 2015). In fact, in our study several immune functions and oxidative stress parameters, which have been previously described as markers of biological age and predictors of longevity (Martínez De Toda et al., 2016, 2020) were impaired in ENPAM groups after the cohabitation for two months with PAM, results that could indicate that some of these parameters, depending on the context, may not be directly related to longevity. Nevertheless, the fewer negative effects observed in ENPAM = 50% and ENPAM > 50% could be due to several reasons. It is necessary to consider that these ENPAM are chronological adults, and they were characterized by their excellent response to a stress situation, such as that represented by the T maze, better than that of regular NPAM, which is related to their longer lifespans (Martínez de Toda et al., 2018). Thus, ENPAM could have been able to develop some hormetic mechanisms as a consequence of the mild-stress produced by the cohabitation with PAM. In fact, previous reports have related the beneficial long-term effects after mild-stress exposure with the possible development of a resilience capacity and thus, causing no negative effects due to the next exposure to a similar stress (Beery and Kaufer, 2015; DeVries, 2002; Russo et al., 2012). Nevertheless, further experiments are needed in order to corroborate these hypotheses, which could explain why these ENPAM groups did not show negative effects on lifespan. In the case of the PAM groups, PAM < 50% exhibited higher mean lifespan than C-PAM, results that seem to be due to an improvement of the nervous and immune systems, supporting the idea that a positive social environment produces beneficial effects on lifespan (Holt-Lunstad et al., 2010; Seeman and Crimmins, 2001; Uchino, 2006). This has previously been described in chronologically old mice that cohabited with adults (Garrido et al., 2018a) and in PAM cohabiting in a predominantly ENPAM environment (Garrido et al., 2019a, 2019b). Nevertheless, PAM < 50% also presented higher mean longevity than PAM > 50%. This seems to indicate that the improvements observed in PAM > 50% do not result in a higher lifespan. Thus, although PAM that cohabite with ENPAM present beneficial effects on regulatory system functions, it is only when PAM live in a predominantly ENPAM environment that positive effects on lifespan occur.

Although we are not certain which mechanisms underlie the effects observed with this cohabitation, we think that these could be related to the physiological characteristics of both experimental groups. In fact, PAM, characterized by their premature aging, could present a decline in their social communication, as has been reported in chronologically old animals (Finkel et al., 2006; Guan and Dluzel, 1994; Osada et al., 2003). Preliminary results in this context seem to indicate that whereas PAM show an altered social behavior, ENPAM exhibit an adequate social response (data not published). Thus, when these PAM and ENPAM cohabited, PAM could have developed mechanisms that improved their social communication. In this sense, visual, olfactory, tactile and auditory perceptions of the presence of ENPAM may generate this improvement, which could enhance the adequate function of regulatory systems, this being stronger when the number of ENPAM increases. Moreover, the beneficial effect of exercise could also underly the improvements observed in PAM when they live in a predominantly ENPAM environment. It is important consider that PAM are classified by the high times that they need to cross the intersection of the T-maze, while ENPAM exhibit low times for this parameter, which can indicate, among other things, higher physical activity in ENPAM in comparison to PAM. Therefore, the cohabitation of both groups could add to the effects of the positive social environment that ENPAM generate, and the possible imitation by PAM of their higher physical activity. In fact, the beneficial effects of exercise have been described in aged mice and in other accelerated and premature aging models (De la Fuente et al.,

2011; Inoue et al., 2017). Another mechanism involved in these results could be the ingestion of fecal bolei, which may alter the microbiota of PAM (Foster et al., 2017; Salchner et al., 2004). In fact, previous studies have described changes in microbiota of animals that live together, producing gastrointestinal tract synchronization, which is due to coprophagy behavior (Deloris et al., 2006; Hildebrand et al., 2013; Hufeldt et al., 2010). Since gut microbiota has been suggested as a potential modulator of regulatory systems, such as the nervous and immune systems (Díaz et al., 2011; Geuking et al., 2014; Sharon et al., 2016), changes in these microorganisms or an increased diversity of them could underlie the effects.

In conclusion, the results of this work reinforce those previously observed in chronologically old mice and in other immunological locations from PAM/ENPAM. Taken together they suggest the relevance of the strategy of cohabitation in improving the functional state of homeostatic systems and consequently the health and lifespan of individuals with chronological and premature aging. It is important to know that all impairments that PAM show at the adult age are natural, since they are accurately detected by the exploration of a new environment like a T-maze and hence the co-housing results shown in the present study could have broader relevance. In fact, most aging studies are performed using genetically modified mice, which live in specific laboratory environments, causing many problems for extrapolation to humans (Briga and Verhulst, 2015; Flatt and Partridge, 2018). However, the results obtained in the case of PAM could be a good model for this extrapolation. Furthermore, this work shows that the proportions of PAM with respect to ENPAM that live together in the cage is very important for many functions and for longevity. Thus, when the number of ENPAM is higher than 50% of the total number of animals in the same cage, PAM exhibit a slowing down of immunosenescence and oxidative stress and show a higher mean lifespan. Although ENPAM seem to show nervous and immune impairments, their lifespan is not affected. In the future, it will be necessary to carry out experiments with slightly different changes in the design to find the type of cohabitation, which would offer positive effects for all individuals.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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