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PREMATURE AGING IN BEHAVIOR AND IMMUNE FUNCTIONS IN TYROSINE HYDROXYLASE HAPLOINSUFFICIENT FEMALE MICE. A LONGITUDINAL STUDY

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Abstract

Aging is accompanied by impairment in the nervous, immune, and endocrine systems as well as in neuroimmunoendocrine communication. In this context, there is an age-related alteration of the physiological response to acute stress, which is modulated by catecholamine (CA), final products of the sympathetic-adreno-medullary axis. The involvement of CA in essential functions of the nervous system is consistent with the neuropsychological deficits found in mice with haploinsufficiency (hemizygous; HZ) of tyrosine hydroxylase (TH) enzyme (TH-HZ). However, other possible alterations in regulatory systems have not been studied in these animals. The aim of the present work was to analyze whether adult TH-HZ female mice presented the impairment of behavioral traits and immunological responses that occurs with aging and whether they had affected their mean lifespan. ICR-CD1 female TH-HZ and wild type (WT) mice were used in a longitudinal study. Behavioral tests were performed on adult and old mice in order to evaluate their sensorimotor abilities and exploratory capacity, as well as anxiety-like behaviors. At the ages of 2±1, 4±1, 9±1, 13±1 and 20±1 months, peritoneal leukocytes were extracted and several immune functions were assessed (phagocytic capacity, Natural Killer (NK) cytotoxicity, and lymphoproliferative response to lipopolysaccharide (LPS) and concanavalin A (ConA)). In addition, several oxidative stress parameters (catalase, glutathione reductase and glutathione peroxidase activities, and reduced glutathione (GSH) concentrations as antioxidant compounds as well as xanthine oxidase activity, oxidized glutathione (GSSG) concentrations, and GSSG/GSH ratio as oxidants) were analyzed. As inflammatory stress parameters TNF-alpha and IL-10 concentrations, and TNF-alpha/IL-10 ratios as inflammatory/anti-inflammatory markers, were measured. Animals were maintained in standard conditions until their natural death. The results indicate that adult TH-HZ mice presented worse sensorimotor abilities and exploratory capacity than their WT littermates as well as greater anxiety-like behaviors. With regards to the immune system, adult TH-HZ animals exhibited lower values of phagocytic capacity, NK cytotoxicity, and lymphoproliferative response to LPS and ConA than WT mice. Moreover, immune cells of TH-HZ mice showed higher oxidative and inflammatory stress than those of WT animals. Although these differences between TH-HZ and WT, in general, decreased with aging, this premature immunosenescence and impairment of behavior of TH-HZ mice was accompanied by a shorter mean lifespan in comparison to WT counterparts. In conclusion, haploinsufficiency of *th* gene in female mice appears to provoke premature aging of the regulatory systems affecting mean lifespan.

Highlights

- TH-HZ adult female mice exhibit behavioral impairment.
- TH-HZ adult female mice show premature immunosenescence.
- TH-HZ female mice present oxidative and inflammatory stress of the immune cells.
- TH-HZ female mice exhibit a significantly shorter mean lifespan.
- Tyrosine hydroxylase haploinsufficiency provokes premature aging.

Keywords

Tyrosine hydroxylase, haploinsufficiency, behavior, immunosenescence, premature aging, mean lifespan, longitudinal study, female mice.

1. Introduction

Aging is characterized by deterioration of the regulatory systems (nervous, endocrine, and immune) and of the communication between them, with this age-related loss of homeostatic response being related to increasing morbidity and mortality (De la Fuente and Miquel, 2009). In the context of neuroendocrine aging, as a consequence of the accumulation of alterations in the nervous cells and progressive modifications in the release of several hormones and neurotransmitters, a decrease of many capacities appears. This is the case with sensorimotor abilities, cognition, memory and attention (Brenes et al., 2008; Greenwood & Parasuraman, 2003; Krampe, 2002; Li and Lindenberger, 2002; Mittenberg et al., 1989; Smith et al., 1999). The age-related changes in the immune system, known as immunosenescence, affect innate and acquired immune responses, producing an increased susceptibility to infectious diseases, autoimmune processes, and cancer (De la Fuente and Bauer, 2016; Gruver et al., 2007). In fact, several immune system parameters have been related to health and the risk of mortality (Dewan et al., 2012; Fulop et al., 2011, Wayne et al., 1990). Moreover, several functional capacities of immune cells have been proposed as markers of the rate of aging of each subject, showing his/her biological age, and as predictors of life expectancy (Martinez de Toda et al., 2016). The age-related physiological impairment seems to be due to the establishment of chronic oxidative and inflammatory stress, provoked by the accumulation of oxidants and inflammatory compounds together with a decrease in the anti-oxidant and anti-inflammatory defenses (De la Fuente and Miquel, 2009).

One inadequate response to stress situations has been considered an example of the loss of cross-talk between regulatory systems (Pedersen et al., 2001) and related to chronological, premature and accelerated aging (Bauer, 2008; Cruces et al., 2014; Gouin et al., 2008; Viveros et al., 2007). In these stress responses two main neuroendocrine pathways are involved: the hypothalamic-hypophysis-adrenal (HPA) axis that releases glucocorticoids (GC) and the sympathetic-adreno-medullary (SAM) axis, characterized by the release of catecholamine (CA). These neurotransmitters and hormones play an important role in organizing the stress response by binding to their receptors in brain and peripheral tissues. In this context, CA are a fundamental part of the maintenance of correct neuroimmunoendocrine communication (Bellinger et al., 2008; Elenkov, 2007; Elenkov et al., 2000). In the nervous system, CA, mainly noradrenaline (NA) and dopamine (DA), are fundamental in a wide

variety of neurological functions, such as cognition, attention, anxiety response, emotion, and memory formation, as well as locomotor control (Borodovitsyna et al., 2017; Foote et al., 1983; Robbins and Everitt, 1995; Sara, 2009). Regarding the immune system, although the effects of CA on immunity are controversial, they are considered key modulators of both innate and acquired immune response (Weinstein et al., 2015). Thus, CA, mainly NA, by β_2-adrenergic receptor (β_2-AR) activation, seem to modify the phagocytosis process (Borda et al., 1998; Garcia et al., 2003), and to inhibit lymphocyte response, e.g. proliferation of T lymphocytes (Elliott et al., 1992) and production of IL-2 (Bartik et al., 1993). They also decrease oxygen radical production (Guirao et al., 1997; Schopf and Lemmel, 1983) and limit the magnitude of both acute and chronic inflammatory responses by shifting the cytokine balance from a pro-inflammatory towards an anti-inflammatory cytokine profile (Vizi and Elenkov, 2002), exerting, in general terms, anti-inflammatory functions (Marino and Cosentino, 2013; Scanzano and Cosentino, 2015). Nevertheless, CA concentrations show an age-related decline in brain as well as in most of the primary and secondary lymphoid organs, changes that may account for the impairment of nervous functions and for the immunosenescence associated with aging (Madden et al., 1997; ThyagaRajan and Priyanka, 2012). In fact, inadequate CA concentrations have been related to neuroinflammation, a typical process of aging (Feinstein et al., 2016). Studies with old rodents have reported decreased Natural Killer (NK) activity as well as a suppression of lymphoproliferation in response to mitogens, changes also related to the decreased CA concentrations that occur with advancing age (Simioni et al., 2007; ThyagaRajan et al., 2011).

Tyrosine hydroxylase (TH) catalyzes the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine, being the first and rate-limiting step of the biosynthesis of CA (Nagatsu et al., 1964). The activity of this enzyme in some cerebral areas and the β-AR responses, decrease with aging (Cotter and O´Malley, 1983; Ponzio et al., 1982; Reymond et al., 1984). In this context, a null mutation in the mouse *th* gene causes profound depletion of CA and lethality of the homozygous mutants from mid-gestation because of cardiovascular failure (Kobayashi et al., 1995; Rios et al., 1999; Vazquez et al., 2014; Zhou et al., 1995). However, hemizygous (HZ) TH mice (TH-HZ) are apparently normal in their development, while displaying a decline in TH activity in their tissues (Kobayashi et al., 1995). Although in these animals several neuropsychological deficits, like alterations in long-term memory and conditioned learning have been observed (Kobayashi et al., 2000), the state of their homeostatic systems has been scarcely studied. The aim of the present work was to determine, in a longitudinal study, whether adult TH-HZ mice

showed an impairment of the regulatory systems similar to those that occur with aging, and if this affects their mean lifespan.

2. Methods

2.1. Animals

We used ICR-CD1 TH-HZ (n=31) and wild type (WT; n=31) virgin female littermate mice. The TH-HZ mice with ICR-CD1 background were from a colony derived in the laboratory of Dr. Flora de Pablo of the Centro de Investigaciones Biológicas as previously described (Vázquez et al., 2014). TH-HZ mice were healthy and with no signs of any associated lesions. The growth rates of these animals were indistinguishable from those of their WT littermates. 5-6 mice were housed per cage, kept separate by genotype after 21 days of birth and maintained under standard animal laboratory conditions of temperature (22±2ºC) and humidity (50-60%), and in a 12/12h reversed light/dark cycle (lights on at 20:00h) to avoid circadian interferences. Mice had access to tap water and standard pellets *ad libitum*. 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 Complutense University of Madrid (Spain). Animals were treated according to the guidelines of the European Community Council Directives 2010/63/EU.

2.1.1. Experimental design

TH-HZ (n=10) and WT (n=10) female mice were used for behavioral tests and the collection of peritoneal leukocytes throughout their lives (longitudinal study). We used female mice due to males showing aggressive and dominant behavior, making it impossible to house them in groups. Moreover, the housing of one male individual per cage causes alterations in the immune system due to isolation stress (Cruces et al., 2014). Peritoneal leukocytes were collected at 2±1 (early young age), 4±1 (late young age), 9±1 (late adult age), 13±1 (mature age), and 20±1 months (old age). The ages of study were selected as key ages of mouse life. Behavioral tests were performed at 6±1 months (early adult age) and 18±1 months (early old age). This design allowed a period of 2-3 months between the last peritoneal leukocyte collection and behavioral tests at adult and old ages. The period of 12 months between both series of behavioral tests, prevented mice from remembering them. Furthermore, CA peritoneal leukocyte contents were determined at 9±1 months, due to this age being considered the adult reference age. In addition, CA plasma concentrations were measured in a separate group of TH-HZ (n=8) and WT (n=8) littermate mice at 9±1

months of age, with the objective of determining if differences exist in CA contents depending on the type of sample. Finally, a third group of animals (n=13 for each genotype) were used to analyze the mean lifespan.

2.2. Behavioral tests

The experiments were performed from 09:00 to 12:00h in accordance with the Spanish legislation on "Protection of Animals Used for Experimental and Other Scientific Purposes" and the European Communities Directives (2010/63/EU) on this subject.

Behavioral testing, performed at the ages of 6±1 (adult) and 18±1 (old) months, took place during three consecutive days. On the first day, animals were subjected to the whole battery of sensorimotor and T-maze tests. On the second day, the holeboard test was performed, and, finally, the elevated plus maze was carried out on the third day. This sequence of testing was based on previous reports by different authors (Giménez-Llort et al., 2002; Johansson et al., 2001). The tests were performed under red light with a white light 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). In order to consider possible interferences in equilibrium, motor coordination and traction capacities due to high weights, as well as ensure that all animals had a similar state of activity previous to carrying out the behavioral tests, all mice were weighed before the first day of these tests. Olfactory trails were removed by cleaning the surface of the apparatus after each mouse.

2.2.1. Sensorimotor abilities

2.2.1.1. Visual placing reflex

The visual placing reflex was performed in order to evaluate the function of the visual system. For this test, the mouse was suspended by the tail and lowered toward a solid black surface. Complete extension of the forelimbs was considered a positive response. The mean response was rated in three trials.

2.2.1.2. Hindlimb extensor reflex

Hindlimb extensor reflex was analyzed during the visual placing reflex test as the ability to perform complete extension of the hindlimbs when the animal was suspended by the tail. Such response was considered positive. The mean response was rated in three trials.

2.2.1.3. Wood rod test

In order to assess motor coordination, the mouse was submitted to the wood rod test, which consists of an elevated (22 cm high) wooden rod (80 cm in length) divided in segments of 10 cm, with a width of 2.9 cm. Mice were placed in the center of the rod for one trial of 60 seconds. Motor coordination was measured by the latency (in seconds) to leave the starting segment and by the total number of segments crossed.

2.2.1.4. Tightrope test

The tightrope test was used to evaluate motor coordination, muscular vigor, and traction in two consecutive trials: one training trial of 5 seconds and a test trial of 60 seconds (Baeza et al., 2010; Guayerbas et al., 2002a; Miquel and Blasco, 1978). The apparatus consists of an elevated (40 cm high) horizontal tightrope (60 cm length divided into 6 segments of 10 cm), which is held by two metallic rods. Mice were hung by their forelimbs in the middle of this tightrope. 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 evaluated by the number of mice that fell off during the trial as well as by the latency (seconds) to fall. Finally, traction was assessed analyzing the different parts of the body that mice used to remain hanging (forelimbs, hindlimbs and tail). The percentage of mice displaying the maximum traction capacity (using forelimbs, hindlimbs, and tail) was also analyzed.

2.2.2. Exploratory and anxiety-like behavioral tests

2.2.2.1. Holeboard test

In order to analyze the "non-goal directed behavior" (evaluated by horizontal and vertical activity) as well as "goal-directed behavior" (analyzed by number and time of head-dipping) of mice, a holeboard test was used. The apparatus consists of a box (60 x 60 x 45 cm) with matte-painted metallic walls. The base was divided into 36 squares (10 x 10 cm), bearing four equally spaced holes (3.8 cm diameter) in the inner zone. We considered the inner zone as the four central squares and the external zone as the 12 squares nearest the walls. Plastic objects were placed in each hole to attract the animal´s attention and drive their "goal-directed behavior". The test was performed for 5 min and the parameters recorded for "non-goal directed behavior" were total locomotion (total number of squares that the animal crosses), percentage of inner locomotion (total number of inner squares that the animal crosses divided by total locomotion) and external locomotion (total number of external squares that the animal crosses divided by total locomotion). All these measurements indicate the horizontal activity of the animal. As vertical activity parameters, the total number of rearing and the time (in seconds) of each rearing were analyzed. Furthermore, the total number of head-dipping and the time (in seconds) of each head-dipping were evaluated as

"goal-directed behavior". Finally, self-grooming and freezing behaviors (number and duration, in seconds, of grooming and freezing) were also recorded.

2.2.2.2. T-maze test

The spontaneous horizontal exploratory behavior of the mice was also tested in a T-shaped maze (short arms: 25 x 10 cm; long arm: 65 x 10 cm; walls: 20 cm high) (Baeza et al., 2010). The mouse was placed inside the "short" arm of the maze with its head facing the 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.

2.2.2.3. Elevated plus maze

The apparatus consists of two opposing open arms (45 x 10 cm) and two enclosed arms (5 x 10 x 50 cm) that extend from a central platform (10 x 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. Total number of entries (four paws criteria) in open arms and in closed arms were recorded. The total number of grooming was also recorded. Finally, the percentage of time spent in open and closed arms, and platform was calculated.

2.3. Collection of peritoneal leukocytes

Peritoneal cellular suspensions were collected between 08:00 and 12:00 h, with the objective of avoiding the possible interference with circadian variations. The use of peritoneal cell samples has the advantage of not having to sacrifice the animals and of being minimally invasive. Non-anesthetized mice were held by cervical skin, the abdomen was cleansed with 70% ethanol, and 3 ml of sterile Hank's solution, previously tempered at 37ºC, was injected intraperitoneally. After abdominal massage, approximately 80% of the injected volume was recovered from the hole previously made by the needle employed for the injection of Hank's solution. Then, the macrophages and lymphocytes from the peritoneal suspensions identified by their morphology were quantified in Neubauer chambers using an optical microscopy (40x). 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 analyzed parameter, as described in the corresponding section.

2.4. Immune function parameters

2.4.1. Phagocytosis of macrophages

The study of the phagocytic capacity of peritoneal macrophages, adjusted to 5 x 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 migration inhibitory factor (MIF) culture plates (Sterilin, Teddington, England) for 30 min. Then, 20 µl of latex beads (1.09 mm diluted to 1% in phosphate-buffered saline solution (PBS), Sigma, St. Louis, MO), pre-washed with PBS, 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 phagocytic efficiency was determined using optical microscopy (100x) and expressed as the number of macrophages with at least one bead ingested per 100 macrophages.

2.4.2. 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 37ºC in a humidified atmosphere of 5% CO_2. Then, they were centrifuged again, and 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.

2.4.3. Lymphoproliferation assay

The lymphoproliferation assay was carried out following a method previously described (Guayerbas et al, 2002c). Resting lymphoproliferation as well as that in response to 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 alone or supplemented with ConA (1µg/ml, Sigma-Aldrich) or LPS (1 µg/ml, Sigma-Aldrich) were added. After 48 h of incubation at 37ºC in a sterile and humidified atmosphere of 5% CO_2, 100 µl of culture supernatants were collected for cytokine measurements. Then, 0.5 µCi [3H] thymidine (MP Biomedicals, Santa Ana, CA, USA) was added to each well and the medium was renewed. The plates were incubated for 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. In the case or resting lymphoproliferation, the results were expressed as counts per minute (c.p.m.). Nevertheless, 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 percentage of stimulation.

2.4.4. Cytokine measurements

Cytokine concentrations, including the pro-inflammatory cytokine TNF-alpha and the anti-inflammatory cytokine IL-10, in supernatant of peritoneal leukocytes cultivated in resting conditions, were measured simultaneously with luminometry using a mouse cytokine/chemokine panel (Milliplex MAP kit, Millipore). Briefly, the filter plate was pre-wet with assay buffer and vacuum filtered before adding 25 µl of standard, control, or experimental samples to the appropriate wells. 25 µl of premixed beads were then added to each well and incubated overnight at 4ºC with shaking. After two washes, 25 µl of detection antibody was added to each well and incubated for 1 h at room temperature and then treated with streptavidin-phycoerythrin (25 µl) for 30 min at room temperature. The plate was then washed twice with wash buffer and vacuum filtered, and, finally, the beads were re-suspended in 150 µl of sheath fluid and the plate was read using a luminometer. The results were expressed as pg/ml of TNF-alpha and IL-10. Also, we calculated TNF-alpha/IL-10 ratio as inflammatory stress marker. Concentrations as low as 2.3 pg/ml for TNF-alfa and 2.0 pg/ml for IL-10 can be detected.

3.5. Oxidative stress parameters

3.5.1. Catalase activity

Catalase activity was determined following a method previously described (Beers & Sizer, 1952), with slight modifications introduced by our research group (Alvarado et al., 2006a). The enzymatic assay was followed spectrophotometrically for 80 seconds at 240 nm by the decomposition of hydrogen peroxide (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.

3.5.2. 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 phosphate (β-NADPH) (6 mM, Sigma-Aldrich) by this enzyme. The reaction was followed by spectrophotometry at 340 nm for 240 seconds. The results were expressed as milliunits (mU) of enzymatic activity per 10^6 peritoneal leukocytes.

3.5.3. Glutathione peroxidase activity

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

3.5.4. 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 reaction that 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 4ºC. Pelleted cells were re-suspended in phosphate buffer containing ethylenediaminetetraacetic acid (EDTA) (0.1 M, pH 8; Sigma-Aldrich). Then, samples were sonicated, and after the addition of 5 µl of perchloric acid ($HClO_4$) (60%; Sigma-Aldrich), they were centrifuged at 9500 g for 10 min at 4ºC. Aliquots of 10 µl of supernatants of immune cells 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 interference of GSH with measurement of GSSG, and then incubated at room temperature for 30 min in the dark. Then, 182 µl of sodium hydroxide (NaOH) (0.1 N; Panreac) and 20 µl of OPT (1mg/ml in methanol) were incorporated and the plate was incubated for 15 min under the same conditions. The fluorescence 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 was added to the 10 µl of cell supernatants dispensed in the wells. The plate was incubated for 15 min under the same conditions, and fluorescence emitted by each well was measured at the same wave-length. The results were expressed as nmol GSH/10^6 peritoneal leukocytes.

3.5.5. 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 H_2O_2, which in the presence of horseradish peroxidase (HRP) 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 (0.05M, pH 7.4) containing EDTA (0.1M, 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 37ºC, 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.

3.6. Catecholamine concentrations

3.6.1. Plasma catecholamine concentrations

Mice were sacrificed and blood was collected immediately in heparinized tubes, and plasma was obtained by centrifugation (1000 g; 20 min). Plasma CA concentrations were analyzed with a commercially ELISA kit (3-CAT

Plasma ELISA$^{high\ Sensitive}$, LDN Labor Diagnostika Nord, Nordhorn). Results were expressed in µg/ml and each sample was assayed in duplicate.

3.6.2. Endogenous leukocyte catecholamine content

Aliquots of 10^6 peritoneal leukocytes/ml Hank's solution, obtained from mice of the longitudinal study at adult age (9±1 months of age), were centrifuged at 1200 g for 10 min at 4ºC. Pelleted cells were re-suspended in 150 µl of HCl buffer (0.01 N, Panreac) in presence of EDTA (1 mM; Sigma-Aldrich) and sodium metabisulfite (4 mM; Sigma-Aldrich). Later, samples were sonicated and centrifuged at 3200g for 20 min at 4ºC. CA concentrations were analyzed in the leukocyte supernatant with a commercially ELISA kit (3-CAT Research ELISA™, LDN Labor Diagnostika Nord, Nordhorn). Results were expressed as µg/10^6 peritoneal leukocytes, and each sample was assayed in duplicate.

3.7. Mean lifespan

In order to evaluate the possible effects of *th* haploinsufficiency on mean lifespan, a group of TH-HZ and WT littermates (n=13) were housed in standard conditions until their natural death.

3.8. Statistical analysis

The data were expressed as the mean ± standard deviation (S.D.) of the values. Statistics were performed using SPSS version 21.0 (Chicago, IL, USA). The normality of the samples was tested with the Kolmogorov-Smirnov test. For qualitative data, the Chi-square test was used. In the case of mean lifespan, the Kaplan-Maier test was used. The data were statistically evaluated with Student's t tests, $p<0.05$ being taken as the minimum significance level. For the immune function parameters and oxidative/inflammatory stress markers, differences over time were assessed by comparing each age to the adult age (9±1 months) of the same genotype.

4. Results

4.1. Plasma catecholamine concentrations

In order to determine the possible effects of *th* haploinsufficiency on circulating CA concentrations, we assessed the CA concentrations in plasma of TH-HZ and WT mice at adult age (9±1 months). Results corresponding to plasma adrenaline (A), NA and DA expressed as µg/ml plasma, are shown in Figure 1 (A, B and C, respectively). TH-HZ mice had significantly lower plasma concentrations of A, NA, and DA than their WT littermates ($p<0.05$, 0.001, 0.05, respectively).

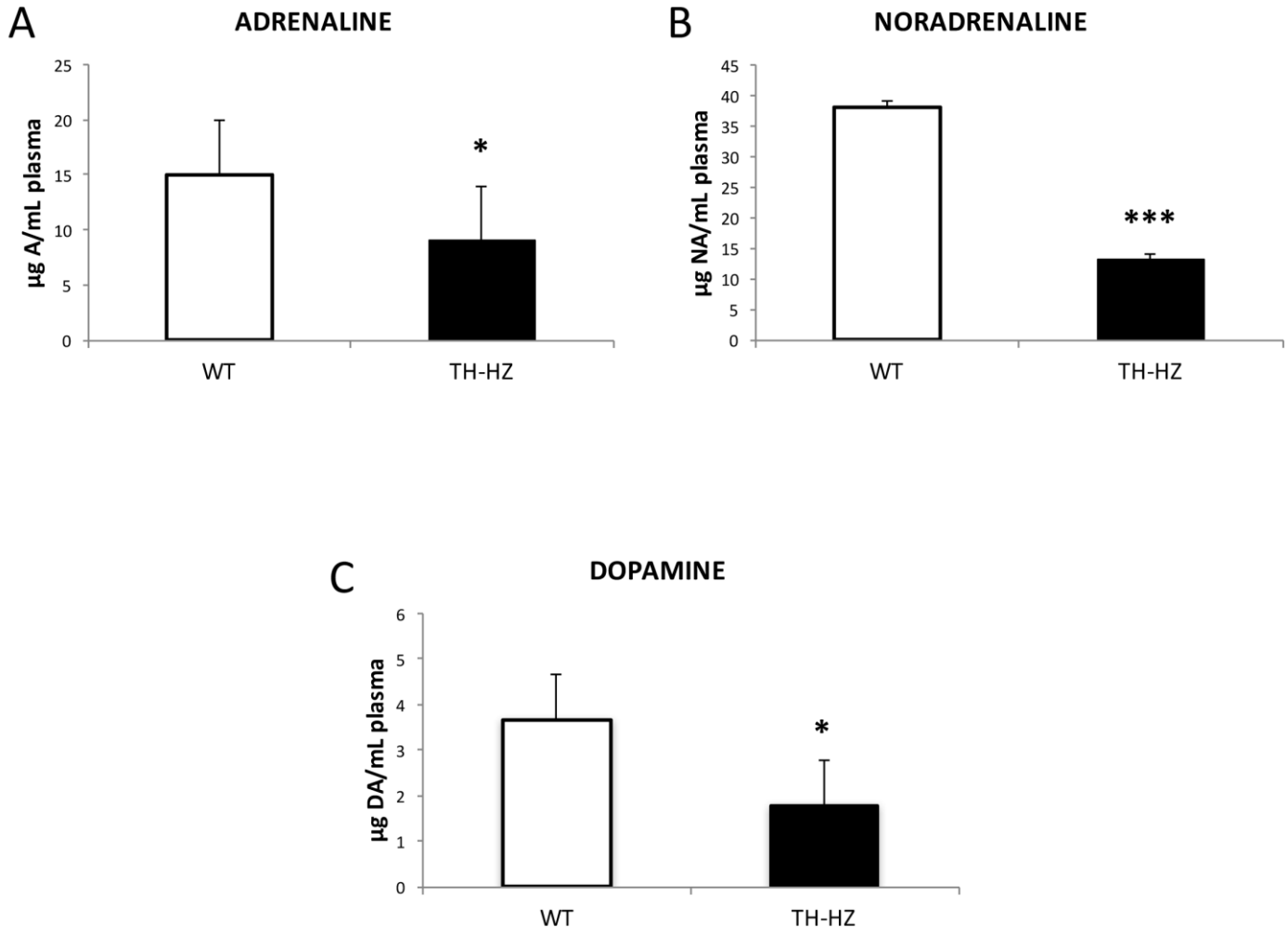

Figure 1. Concentrations of catecholamine in plasma. Adrenaline (A), noradrenaline (B) and dopamine (C) concentrations (µg/ml) in plasma of adult TH-HZ and WT mice. Each column represents the mean ± standard deviation of values corresponding to 8 animals. * $p<0.05$, *** $p<0.001$ with respect to WT group.

4.2. Behavioral tests

Tables 1 and 2 summarize the results obtained in the behavior parameters analyzed in TH-HZ and WT mice at adult and old ages.

4.2.1. Sensorimotor abilities

The results corresponding to sensorimotor abilities are summarized in Table 1. With respect to motor coordination evaluated in the wood rod test, adult TH-HZ mice showed a higher latency to leave the starting segment than WT at the same age ($p<0.001$), maintaining these differences in old age. Similarly, in the tightrope test, adult TH-HZ mice also presented greater latency to leave the starting segment than the adult WT mice ($p<0.01$). However, while the WT mice increased the value of this parameter with age ($p<0.01$), the TH-HZ mice did not do so, reaching similar values at old age. In relation to muscular vigor, adult TH-HZ mice exhibited lower

latency to fall in the tightrope test than their WT littermates ($p<0.01$). With age, WT and TH-HZ mice decreased their latency to fall ($p<0.05$ and $p<0.001$, respectively) maintaining the difference observed at the adult age ($p<0.001$). The results described up to here suggest that the adult TH-HZ mice present premature impairment of motor coordination and muscular vigor compared to their WT littermates.

4.2.2. Exploratory and anxiety-like behavior tests

Table 2 summarizes the results corresponding to anxiety-like behaviors and exploratory capacity. Regarding anxiety-like behaviors evaluated using the elevated plus maze, adult TH-HZ mice presented a lower total number of entries (Table 2) as well as the percentage of time in open arms (Fig. 2A) than their WT counterparts ($p<0.05$ and $p<0.001$ respectively). These results could show increased anxiety-like behavior in adult TH-HZ mice. Also, with age, WT mice decreased the total number of entries in the open arms ($p<0.05$), reaching values similar to those of the TH-HZ mice, which did not change with aging. With respect to self-grooming behavior in the elevated plus maze, adult TH-HZ animals presented a higher total number of events than their WT littermates ($p<0.001$) and this difference was maintained in old age ($p<0.001$). Similarly, self-grooming behavior in the holeboard test showed that the adult TH-HZ mice presented a higher total number and extension of grooming events than their WT littermates ($p<0.001$) (Table 2 and Fig. 2B). In addition, the total number and extension of the freezing events in adult TH-HZ mice were higher than that of their WT littermates ($p<0.001$). With age, the WT mice increased the total number of freezing events ($p<0.001$) as well as grooming and freezing times ($p<0.001$ and $p<0.05$, respectively), reaching values similar to those of the TH-HZ mice, which did not change with age (Table 2).

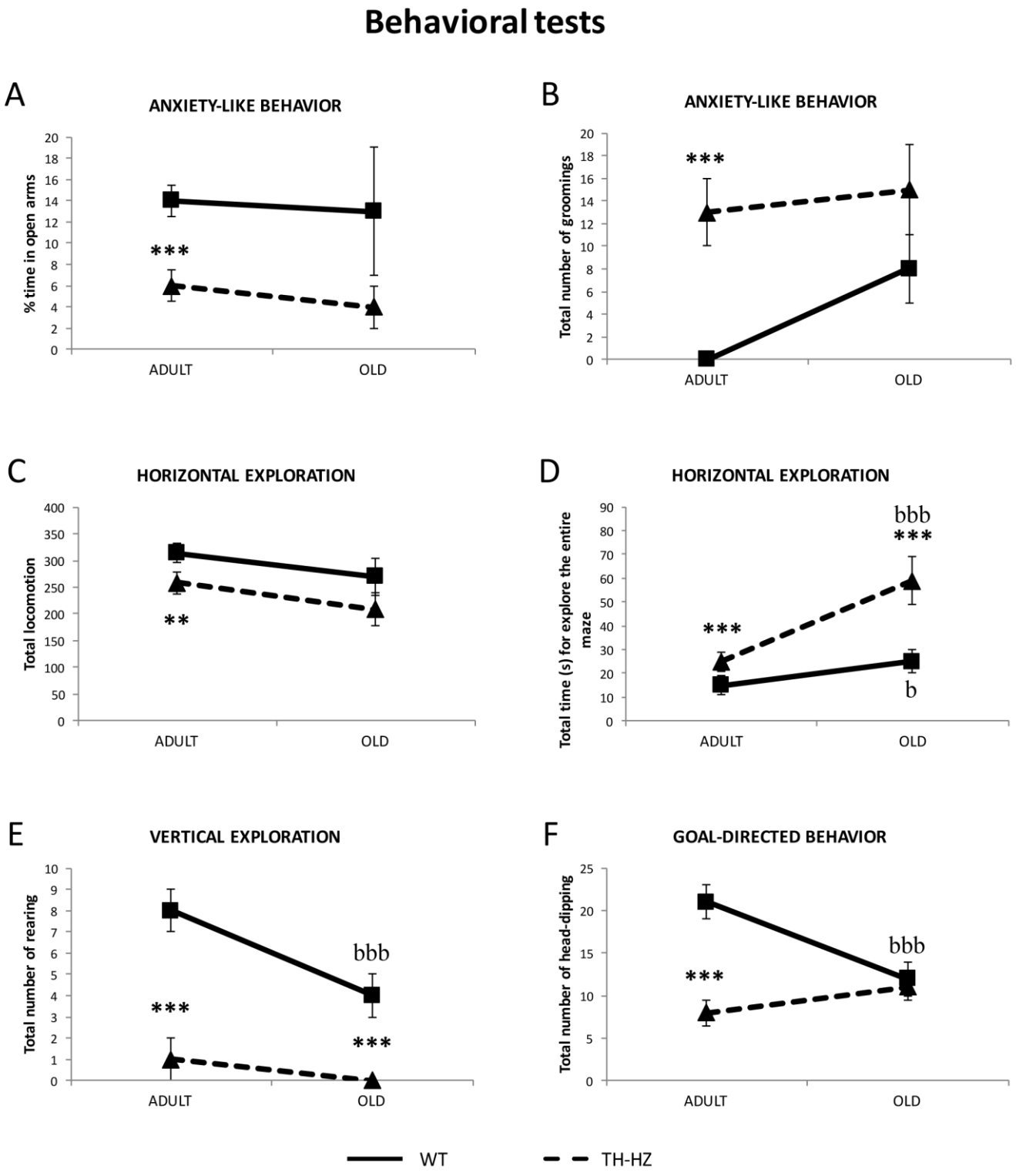

Figure 2. Exploratory and anxiety-like behaviors. Percentage (%) of time in open arms in elevated plus maze (A), total number of grooming events in holeboard test (B), total locomotion in holeboard test (C), total time (s) of exploration of the T maze (D), total number of rearing events in holeboard test (E), and total number of head-dipping events in holeboard test (F) evaluated in TH-HZ and WT mice - at 6±1 months (adult) as well as at 18±1 months (old). Each point represents the mean ± standard deviation of values corresponding to 10 animals. **

p<0.01, *** p<0.001 with respect to WT of same age group; b p<0.05, bb p<0.01, bbb p<0.001 with respect to value obtained in adult age of the same genotype.

In relation to exploratory behavior assessed in the holeboard test, adult TH-HZ mice showed lower total locomotion (p<0.01) (Fig. 2C) than the adult WT animals, which is indicative of lessened horizontal exploration. Moreover, the percentage of inner locomotion, indicative of exploratory capacity, decreased in the old WT mice (Table 2) (p<0.001). On the contrary, the percentage of external locomotion was higher in the adult TH-HZ mice than in their WT littermates (p<0.001), which represents a greater anxiety-like behavior. This parameter increased with age in WT mice (p<0.001) and remained similarly elevated in the aged TH-HZ mice. In addition, the crossing time of the intersection of the T-maze was higher for the adult TH-HZ mice than for their WT counterparts (p<0.001), increasing in both genotypes with age (p<0.001) and therefore maintaining the difference between TH-HZ and WT mice in old age (p<0.001). The total explore time for the entire T-maze was greater for the adult TH-HZ mice than for the WT group (p<0.001) and increased significantly with age in both genotypes (p<0.05 and p<0.001 for WT and TH-HZ, respectively) (Fig. 2D). The differences between both genotypes were maintained in old age (p<0.001). Regarding vertical activity, adult TH-HZ mice showed a lower total number and duration of rearing events in the holeboard test (Fig. 2E) than the WT mice (p<0.001). Further, with age, WT mice exhibited a decrease in both parameters (p<0.001). Finally, with respect to goal-directed behavior evaluated in the holeboard test, adult TH-HZ mice exhibited a lower total number of head-dipping events (Fig. 2F) than their WT littermates (p<0.001), this parameter decreasing in older WT mice (p<0.001) whereas the values in TH-HZ group were maintained. Similarly, with respect to the total time of head-dipping, adult TH-HZ mice showed lower values than the WT group at the same age (p<0.001) and a decrease with age in the latter (p<0.001) was also observed. Old TH-HZ mice also showed a lower time of head-dipping with respect to old WT mice (p<0.001). Although, this parameter did not change in the TH-HZ with age, it remained lower in the old TH-HZ than in the old WT (p<0.001).

4.3. Immune function parameters

The longitudinal study of several immune function parameters in peritoneal leukocytes was carried out at 2±1, 4±1, 9±1, 13±1, and 20±1 months, and the results are shown in Figure 3.

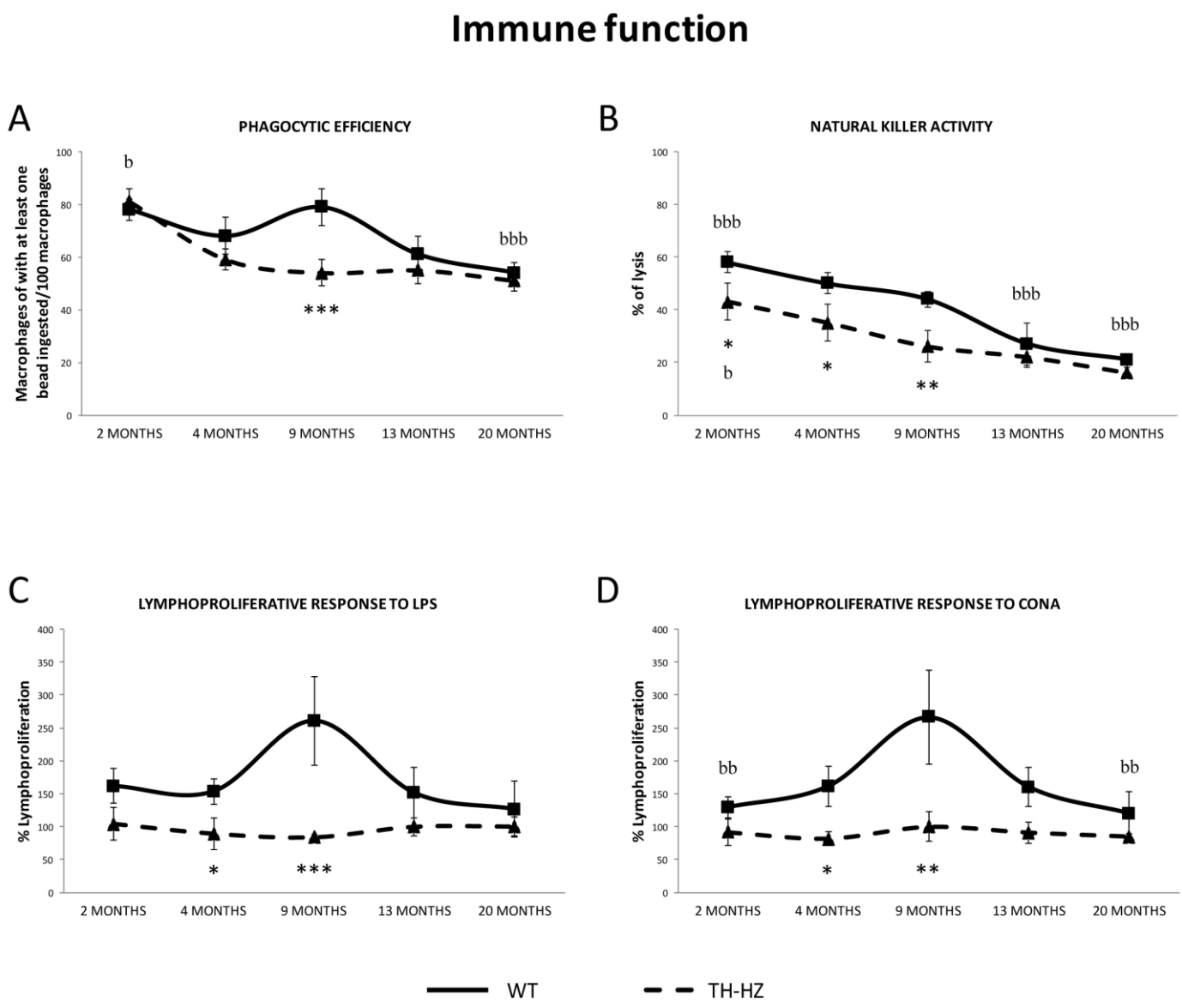

Figure 3. Immune functions. (A) Phagocytic efficiency (%), (B) Natural Killer cytotoxicity (% lysis of tumor cells), (C) lymphoproliferative response to LPS (%), (D) lymphoproliferative response to ConA, evaluated in peritoneal leukocytes from TH-HZ and WT mice at 2±1, 4±1, 9±1, 13±1, and 20±1 months of age. Each point represents the mean ± standard deviation of values corresponding to 10 animals, with each value being the mean of triplicate or duplicate assays. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ with respect to WT group at same age; b $p<0.05$, bb $p<0.01$, bbb $p<0.001$ with respect to adult age (9±1 months) of the same genotype.

4.3.1. Phagocytic efficiency

TH-HZ mice had a lower phagocytic efficiency at 9 months of age than their WT counterparts ($p<0.001$) (Fig. 3A). WT mice presented a decreased phagocytic efficiency at old age with respect to the adult age ($p<0.001$),

however the phagocytic efficiency of the TH-HZ group only was higher at 2 months of age with respect to the adult age ($p<0.05$).

4.3.2. Natural killer cytotoxicity

TH-HZ mice showed lower values for this parameter from early young to adult age [2±1 ($p<0.05$), 4±1 ($p<0.05$), and 9±1 ($p<0.01$) months], being similar at mature and old ages (13±1 and 20±1 months) to that of their WT littermates (Fig. 3B). The longitudinal study of the WT group showed elevated levels of NK cytotoxicity at 2±1 months respect to 9±1 months ($p<0.001$) and diminished values at 13±1 and 20±1 months respect to 9±1 months ($p<0.001$). Only in the TH-HZ mice, we observe an increase in NK cytotoxicity at 2±1 months ($p<0.05$) with respect to values obtained at adult age.

4.3.3. Lymphoproliferative response

In general, TH-HZ mice exhibited a trend to lower lymphoproliferative response to LPS than WT animals at all ages, with statistically significant differences at 4±1 months ($p<0.05$) and 9±1 months ($p<0.001$) (Fig. 3C). Similarly, TH-HZ mice showed a trend to lower values of proliferative response to ConA than WT animals at all ages, with statistical differences at 4±1 months ($p<0.05$) and 9±1 months ($p<0.01$) of age (Fig. 4D). At 2±1 months as well as 20±1 months, the WT group presented a decreased lymphoproliferative response to ConA in comparison to adult age (9±1 months; $p<0.01$). These differences were not observed in TH-HZ mice.

4.4. Parameters of oxidative and inflammatory stress and resting lymphoproliferation

4.4.1. Parameters of oxidative stress

4.4.1.1. Antioxidants

The antioxidants evaluated in peritoneal leukocytes were the activities of catalase, glutathione reductase, and glutathione peroxidase as well as concentrations of reduced glutathione. The results for these parameters are shown in Table 3 and Figure 4.

4.4.1.1.1. Catalase activity

Although TH-HZ mice showed a trend to lower catalase activity than their WT littermates at all ages, these differences were statistically significant only at 2±1 months ($p<0.001$) (Table 3). In relation to differences due to age, the WT group presented an increased catalase activity at 2±1 months with respect to values obtained at adult

age (9±1 months). No differences were observed in the TH-HZ group along the studied period respect to their adult age.

4.4.1.1.2. Glutathione reductase activity

The TH-HZ mice exhibited a lower glutathione reductase activity than WT animals at all ages in the study, showing statistically significant differences at 4±1 months ($p<0.001$), 9±1 months ($p<0.001$), 13±1 months ($p<0.001$) and 20±1 months ($p<0.001$). No differences were found due to age in either genotypes (Fig. 4A).

4.4.1.1.3. Glutathione peroxidase activity

Although at 2±1 months TH-HZ mice exhibited a higher antioxidant activity of this enzyme than their WT littermates ($p<0.001$), at 4±1 months TH-HZ mice presented lower activity than the WT group ($p<0.001$), keeping this tendency at older ages (Table 3). Furthermore, WT as well as TH-HZ mice presented diminished glutathione peroxidase activity at 2±1 months ($p<0.01$, $p<0.001$, respectively) and 4±1 months ($p<0.001$) than at adult age (9±1 months).

4.4.1.1.4. Reduced glutathione concentration

GSH concentration showed a trend to lower in TH-HZ than in WT mice, being statistically significant at 4±1 months ($p<0.001$) and 9±1 months ($p<0.001$) (Fig. 4B). With respect to differences due to age, the WT group presented a decreased GSH concentration at 2±1 months ($p<0.05$), 13±1 months ($p<0. 01$), and 20±1 months ($p<0.001$) than at the adult age. No differences due to age were found in the TH-HZ group.

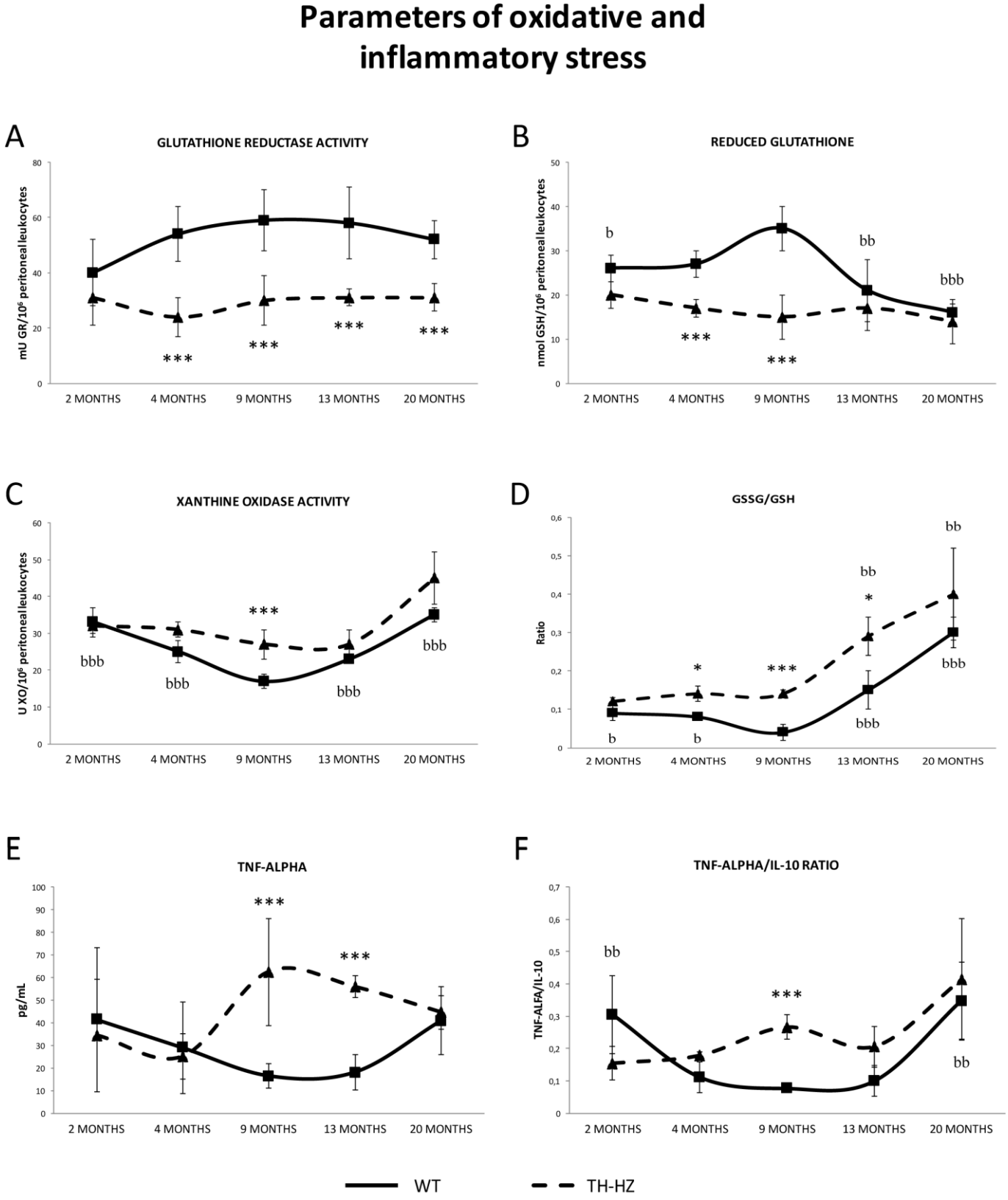

Figure 4. Parameters of oxidative and inflammatory stress. (A) Glutathione reductase activity (mU/10^6 peritoneal leukocytes), (B), Reduced glutathione concentration (nmol/10^6 peritoneal leukocytes), (C) Xanthine oxidase activity (U/10^6 peritoneal leukocytes), (D) GSSG/GSH ratio, (E) TNF-alpha concentration (pg/mL) in resting lymphoproliferation, and (F) TNF-alpha/IL-10 ratio, evaluated in peritoneal leukocytes from TH-HZ and WT mice at 2±1, 4±1, 9±1, 13±1, and 20±1 months of age. Each point represents the mean ± standard deviation of values

corresponding to 10 animals, with each value being the mean of triplicate or duplicate assays. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ with respect to WT group at same age; b $p<0.05$, bb $p<0.01$, bbb $p<0.001$ with respect to adult age (9±1 months) of the same genotype.

4.4.2.2. Oxidants

As oxidant compounds, xanthine oxidase activity as well as oxidized glutathione (GSSG) concentrations were analyzed in peritoneal leukocytes. Also, the GSSG/GSH ratio was calculated as oxidative stress index. These results are presented in Table 3 and Figure 4.

4.4.2.2.1. Xanthine oxidase activity

TH-HZ animals presented a trend to higher XO activity than WT mice, showing statistically significant differences at 9±1 months ($p<0.001$) (Fig. 4C). Longitudinal analysis in the WT mice showed an increase in XO activity at 2±1 ($p<0.001$), 4±1 ($p<0.001$), 13±1 ($p<0.001$), and 20±1 ($p<0.001$) months. Nevertheless, age differences were not found in TH-HZ group.

4.4.2.2.2. Oxidized glutathione concentrations

TH-HZ group presented a trend to higher value for this parameter at all ages of study in comparison to the WT group, with statistically significant differences at 20±1 months of age ($p<0.05$) (Table 3). The WT mice showed an increase in the GSSG concentration at 2±1 months ($p<0.01$), 13±1 months ($p<0.01$), and 20±1 months ($p<0.001$) respect to the adult age. Similarly, TH-HZ animals had increased GSSG concentration at 2±1 ($p<0.05$), 13±1 ($p<0.001$), and 20±1 months ($p<0.001$) respect to adult age.

4.4.2.2.3. GSSG/GSH ratio

TH-HZ mice showed a higher GSSG/GSH ratio than WT animals at 4±1 ($p<0.05$), 9±1 ($p<0.001$), and 13±1 months ($p<0.05$) (Fig 4D). Furthermore, with respect to the values obtained at adult age (9±1 months), the WT group exhibited an increased ratio at 2±1 ($p<0.05$), 4±1 ($p<0.05$), 13±1 ($p<0.01$) and 20±1 months ($p<0.001$). In the case of the TH-HZ group, the increase in GSSG/GSH ratio was observed at 13±1 ($p<0.01$) and 20±1 months ($p<0.01$).

4.4.2.3. Parameters of inflammatory stress

The inflammatory stress parameters evaluated in the longitudinal study (at 2±1, 4±1, 9±1, 13±1, and 20±1 months) were the levels of TNF-alfa and IL-10 cytokine released in resting cultures of peritoneal leukocytes. TNF-alfa/IL-10 ratios were also calculated. The results are presented in Table 3 and Figure 4.

4.4.2.3.1. Cytokine concentrations and TNF-alpha/IL-10 ratios

TH-HZ mice exhibited higher TNF-alpha concentration than WT at 9±1 ($p<0.001$) and 13±1 months ($p<0.01$) (Fig. 4E). In the case of IL-10, TH-HZ mice showed higher concentration than WT at 9±1 months ($p<0.01$) (Table 3). With respect to age, WT showed decreased IL-10 concentration at 20±1 months ($p<0.01$) in relation to values obtained at adult age. Regarding TFN-alpha/IL-10 ratio (Fig. 4F), TH-HZ animals showed higher ratio than their WT littermates at 9±1 months of age ($p<0.001$), and the longitudinal analysis showed increased TNF-alpha/IL-10 ratio at 2±1 ($p<0.01$) and 20±1 months ($p<0.01$) respect to 9±1 months in the WT mice, whereas no age-related differences were found in the TH-HZ group.

4.4.3. Resting lymphoproliferation

With the objective of evaluating the possible early establishment of low-grade inflammation, characteristic of advanced ages, we assessed the proliferative rate of resting (without stimuli) peritoneal lymphocytes. TH-HZ mice displayed higher resting lymphoproliferative rate than the WT animals at 2±1 ($p<0.05$), 4±1 ($p<0.01$) and 9±1 months ($p<0.001$) (Table 3). The longitudinal analysis showed that WT peritoneal lymphocytes presented increase proliferation at 2±1 ($p<0.001$), 4±1 ($p<0.05$), 13±1 ($p<0.05$) and 20±1 months ($p<0.001$) in comparison to values presented at adult age. In the case of the TH-HZ group, the decrease was only observed at 20±1 months ($p<0.05$).

4.5. Catecholamine peritoneal leukocyte content

Since the many of the differences in immune function parameters were observed at 9 months of age, the CA content of peritoneal leukocytes (μg /10^6 peritoneal leukocytes) was evaluated in this age. TH-HZ mice showed lower values of A, NA, and DA concentrations than those obtained in WT animals ($p<0.001$) (Fig. 5).

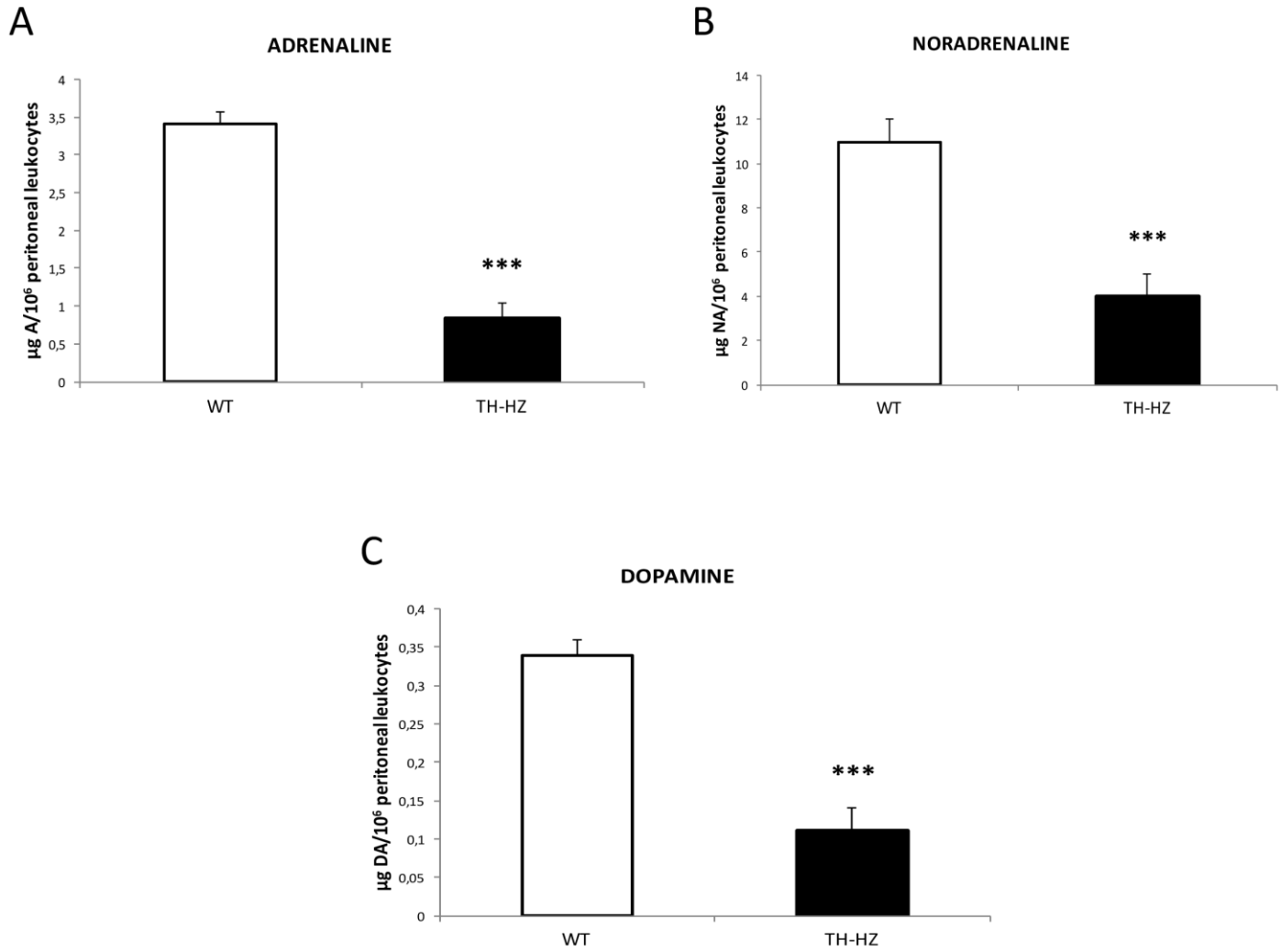

Figure 5. Catecholamine leukocyte contents (µg /10^6 peritoneal leukocytes) at adult age (9±1 months). Adrenaline (A), noradrenaline (B) and dopamine (C) concentrations in TH-HZ as well as WT mice. Each column represents the mean ± standard deviation of values corresponding to 10 animals. *** $p<0.001$ with respect to the WT group.

4.6. Mean lifespan

The mean lifespan of the TH-HZ and WT littermates were analyzed in separate experimental groups designed for that propose. The animals were maintained under standard housing conditions until their natural death. As shown in Figure 6, TH-HZ mice exhibited a shorter mean lifespan (97 *versus* 111 weeks) than their WT counterparts ($p<0.05$).

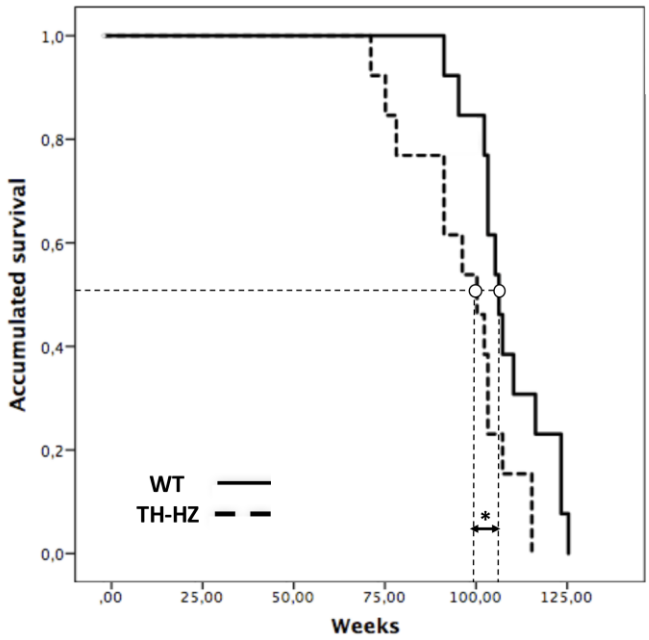

Figure 6. Mortality records of TH-HZ and WT mice (N=13) from youth until the natural death of the animals. * $p<0.05$ with respect to the value in WT mice.

5. Discussion

This research is the first about the effects of a *th* gene haploinsufficiency on behavior and immune cell parameters, throughout the life of mice. Female mice were specifically employed in this longitudinal study since they show good social behavior in comparison to males, which tend to be very aggressive. The results allowed us to propose this genetic alteration in female ICR-CD1 mice as a model of premature aging.

Since the TH enzyme controls a limiting step of CA synthesis, TH-HZ mice are expected to show a decrease in CA concentrations. However, the authors who developed this genetic model with a C57/bL6 background and other later studies have described no differences with respect to control animals both in plasma and brain CA concentrations (Gamella-Pozuelo et al., 2017; Kobayashi et al., 1995; Zhou et al., 1995). Nevertheless, another study has shown lower concentrations of NA in different brain areas (Kobayashi et al., 2000) and our results show that adult TH-HZ mice presented lower plasma concentrations of the three CA analyzed in comparison to WT littermates. These discrepancies could be due to several causes, such as the different strains used in the studies (C57/bL6 vs ICR-CD1) or the *th* gene expression regulation, which seems to vary depending on the genetic background of the mice (Marcel et al., 1998). Since this is higher in the C57/bL6 than in the ICR-CD1 background (Cambon et al., 2010), it could justify possible differences due to the haploinsufficiency between

C57/bL6 and ICR-CD1 animals. In addition, sex differences could also influence the CA circulating values and whereas in the previous studies males were used, in the present work we only employed female mice.

A simple way to evaluate nervous system functions is by performing behavioral tests (Dellu et al., 1994; Gilad and Gilad, 2000). For this reason, TH-HZ mice were subjected to a wide battery of behavioral tests in order to evaluate sensorimotor abilities and exploratory capacity, as well as anxiety-like behavior. The results showed that adult TH-HZ mice presented a decline of motor coordination and muscular vigor, in comparison to their WT counterparts. Exploratory capacity was also altered in the adult TH-HZ mice. These animals showed lower vertical and horizontal exploration as well as reduced goal-directed behavior. Furthermore, anxiety-like behavior was greater in these mice than in the WT at adult age. These results observed at adult age could be due to the lower content of CA since these behavior parameters are linked to catecholaminergic function, mainly to DA and NA actions (Borodovitsyna et al., 2017; Emerich et al., 1993; Foote et al., 1983; Sara, 2009; Robbins and Everitt, 1995). In line with these observations, other studies of adult animals with CA depletion due to TH deficiency showed altered exploratory behavior and sensorimotor abilities, such as lower motor coordination (Korner et al., 2015; Sabbar et al., 2012). Furthermore, those behavioral capacities showed age-related decline in the WT mice, as has been described previously (Boguszewski and Zagrodzka, 2002; Forster et al., 1996; Ingram et al., 1981; Lamberty and Gower, 1992; Thompson, 2008). Nevertheless, we did not observe a further decline with age in the TH-HZ mice, most likely, because they already presented a clear impairment at adult age. In the case of anxiety-like behavior, which increased in TH deficient animals and in old individuals (Faggliani et al., 2015; Morgan et al., 2015; Tinakoua et al., 2015), this was higher in TH-HZ than in WT mice. Thus, in general, adult TH-HZ mice performed similarly to old WT mice for most of the tests. This premature impairment in the behavioral parameters has also been shown in two other models of premature aging, the prematurely aging mice (PAM) (Guayerbas et al., 2002a; Viveros et al., 2001) and the senescence-accelerated mice prone (SAMP) (Aoyama et al., 2013; Markowska et al., 1998; Niimi and Takahashi, 2014). Thus, TH-HZ mice showed markers of premature nervous system aging, a consequence of *th* gene haploinsufficiency.

Regarding the immune functions, concretely those of the innate immunity, TH-HZ mice showed lower phagocytic capacity and NK cytotoxicity than their WT littermates at adult age. Similar results have been described in a preliminary previous study with male TH-HZ ICR-CD1 mice at adult age (Garrido et al., 2017). In addition, these results are in agreement with studies employing TH inhibitors, such as alpha-methyl-p-tyrosyne (alpha-MT) in

splenic leukocytes (Won and Lin, 1989). Several studies have demonstrated a modulator effect of CA on immune functions although with controversial results (Bellinger et al., 2008; Lang et al., 2003; Madden, 2017; Nance and Sanders, 2007). While some authors have reported an inhibitory effect of CA on phagocytic capacity (Borda et al., 1998), others have described stimulatory effects of CA on this immune function (Garcia et al., 2003). These opposite effects could depend on the concentrations of the neurotransmitter present. With respect to lymphoproliferative response, TH-HZ mice showed lower lymphoproliferation in response to LPS and ConA, specific mitogens for B and T lymphocytes, respectively, in comparison to their WT counterparts at adult age. Similarly, adult male TH-HZ mice with background ICR-CD1 have exhibited lower lymphoproliferative response in presence of LPS (Garrido et al., 2017). These results could also be the consequence of the modulatory effects of CA on proliferative response to lymphocytes (Benarroch, 2009; Del Rey and Besedovsky, 2008; Puerto et al., 2005). In fact, following sympathectomy using 6-hydroxydopamine (6-OHDA), a neurotoxin able to deplete peripheral NA nerve fibers, adult animals presented lower mitogenesis induced by LPS or ConA (Delrueperollet et al., 1995; Pacheco-López et al., 2003). Furthermore, all these immune functions studied show an age-related decline in WT animals as can be observed in the present study and in previous reports (Arranz et al., 2010; Douziech et al., 2002; Ferrández et al., 1999; Frasca et al., 2003; Martinez de Toda et al., 2016; Pawelec, 2006; Pawelec et al., 2002; Weinberger, 2017). Nevertheless, no differences were observed due to age in TH-HZ mice, which could be due to the establishment at adult age of a premature immunosenescence. Similar results have been shown in PAM (Alvarado et al., 2006b; Guayerbas et al., 2005; 2002a; 2002b; 2002c; Pérez- Álvarez et al., 2005; Puerto et al., 2002) and in SAMP (Abe et al., 1994; Kumagai et al., 2000; Takeda et al., 1991). This premature immunosenescence could be a consequence of the lower intracellular content of CA in the immune cells of these mice. In fact, endogenous leukocyte CA are fundamental to correct the immune function mediated via autocrine/paracrine AR signaling that regulates the intracellular cAMP levels (Bergquist et al., 1994; Cosentino et al., 1999; Madden, 2017; Qiu et al., 2004). In this sense, lymphocytes treated with α-MT and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), another inhibitor of TH, depressed proliferation of T cell hybridoma in a dose-dependent manner (Tsao et al., 1998).

It is known that a cause of immunosenescence is oxidative and inflammatory stress (De la Fuente and Bauer, 2016). Since oxidative stress is produced by an imbalance between oxidants and antioxidants, with an increase of the first, we measured different antioxidant defenses and oxidant compounds in peritoneal leukocytes of

TH-HZ mice. In general, these cells of adult animals presented lower antioxidant defenses and higher oxidant compounds than the leukocytes of their WT littermates. This oxidative stress due to the haploinsufficiency of *th* appears to be related to the lower concentrations of CA. Similar results have been previously described in adult male TH-HZ mice (Garrido et al., 2017). In addition, although several authors have described the oxidant capacity of CA due to their auto-oxidation generating an increase in the reactive free radicals (Creveling et al., 1975; Graham et al., 1978; Rosenberg, 1988), this capacity seems to be concentration-dependent. In this context, CA show an oxidant capacity at abnormally elevated concentrations (Noh et al., 1999; Sofic et al., 2001), while at physiological amounts, NA and DA exhibit antioxidant capacities (Liu and Mori, 1993; Miura et al., 1996; Noh et al., 1999; Sofic et al., 2001). Thus, the oxidative stress shown in TH-HZ peritoneal leukocytes at adult age could be a consequence of the low plasma and endogenous leukocyte CA concentrations observed in these animals. Furthermore, there is an age-related antioxidant defense decrease and oxidant increase leading to an oxidative stress (Dröge, 2003; Romano et al., 2010; Vida et al., 2014). This fact has been observed in the WT mice of the present longitudinal study. Nevertheless, as the consequence of the early oxidative stress established in TH-HZ animals, no differences were observed in these mice with respect to their WT counterparts at old age. In addition, the inflammatory stress is produced by an imbalance between pro-inflammatory and anti-inflammatory compounds, and in this work we analyzed the amount of TNF-alpha, a typical pro-inflammatory cytokine, and that of IL-10, an anti-inflammatory cytokine, released in resting cultures of peritoneal leukocytes. TNF-alpha levels were higher in culture supernatants of leukocytes from TH-HZ mice than in those of WT at adult age. As it has been shown CA, through increasing intracellular cAMP levels, can inhibit, in leukocytes, the synthesis of TNF-alpha and its release (Guirao et al., 1997; Schopf and Lemmel, 1983). Therefore the lower CA concentrations observed in TH-HZ leukocytes may be responsible for the increased TNF-alpha concentrations. Unexpectedly, the levels of the cytokine IL-10 were higher in the TH-HZ than in the WT resting leukocytes at adult age. These could represent a compensatory mechanism of peritoneal leukocytes against the increased content of pro-inflammatory cytokines. Nevertheless, the TNF-alpha/IL-10 ratio was still higher in the leukocytes of adult TH-HZ mice than in those of WT. This imbalanced pro-inflammatory/anti-inflammatory ratio may indicate a low-grade of sterile inflammation, which is typical of aging (De Martinis et al., 2006; Franceschi and Bonafé, 2003; Franceschi et al., 2000; Martinez de Toda et al., 2017). Indeed, the greater proliferation in resting conditions found in leukocytes of adult TH-HZ also indicates the presence of a sterile inflammation. This oxi-inflamm-aging has also been observed in PAM (Alvarado et al., 2006a;

2006b; Guayerbas et al., 2005; 2002a) and in SAMP (Farr et al., 2003; Kurokawa et al., 2001; Nomura et al., 1989; Okatani et al., 2002; Sureda et al., 2006; Yasui et al., 2003) at adult age. Thus, adult female TH-HZ mice appear to present the establishment of a premature oxi-inflamm-aging, which could be due to their lower CA concentrations in immune cells and plasma.

In mammals, the immune system is relatively immature at birth and completes its development at the end of the individual growth period, which in humans corresponds to the beginning of the second decade of life (Giedd, 2004; Simon et al., 2015; Sowell et al., 1999). In mice, the maturation of the immune system is similar to humans and it is complete on reaching adult age. For this reason, several immune functions may show similar values at young (inmature) and old (senescent) ages (De la Fuente et al., 2003). This fact was also observed in the lympoproliferative response to mitogens in cells of WT mice. In this function and in NK cytotoxicity, the young (2 or 4 months of age) TH-HZ demonstrated poorer performance than their WT littermates. Moreover, this very early immune impairment has also been observed in young PAM (Alvarado et al., 2005; 2006b; Guayerbas and De la Fuente, 2003).

Since the immune parameters evaluated in this study have been proposed as markers of biological age and predictors of longevity (Martinez de Toda et al., 2016), the premature immunosenescence observed in TH-HZ mice could coexist with a short mean lifespan. In agreement, TH-HZ mice had a shorter mean lifespan than their WT counterparts. This shorter mean lifespan seen has also been observed in PAM (Guayerbas et al., 2002a; Martinez de Toda et al., 2016). However, a previous study using male TH-HZ mice (Gamella-Pozuelo et al., 2017), with a different genetic background (C57/bL6), showed opposite effects on the mean lifespan to those described here for the ICR-CD1 female animals. As mentioned above, the mouse strain may influence the *th* gene expression regulation and therefore the CA concentrations. Thus, in the study by Gamella-Pozuelo and cols (2017) these concentrations were normal. We think that this fact together with the use of different sex and background, could explain the different results found in the lifespan of animals. In fact, previous studies in other premature aging mice models such as SIRT1, SIRT2 and SIRT6 deficient mice, have described that survival can be dependent on the genetic background of the strain as well as on the gender. Thus, whereas SIRT1 deficient mice with a 129/SvEv/C57bL6 background died at perinatal or early postnatal stages, mice with a 129/CD1 mixed background more often survived to adulthood (McBurney et al., 2003; Sebastian et al., 2012). In addition, in the case of SIRT6 deficient mice, the mutant female 129/SvJ/BALB/c mice present a significantly increased survival with respect to

the corresponding male, results that indicate the importance of sex in these types of studies (Peshti et al., 2017). Nevertheless, further research is needed to clarify this issue.

In conclusion, the present study suggests that TH-HZ mice (ICR-CD1 female) showed premature alteration of several behavioral capacities and immune functions together with premature oxi-inflamm-aging. This could be a consequence of low CA concentrations due to *th* haploinsufficiency. Since nervous and immune system impairment has been related to loss of cross-talk between regulatory systems as well as to loss of homeostasis and consequently of health, TH-HZ mice could have disrupted neuroimmunoendocrine communication, which impacted on their lifespan. In this sense, NK cytotoxicity has been proposed as a sensible marker of the imbalance of the neuroimmunoendocrine system (Fiserová et al., 2002; Mocchegiani and Malavolta, 2004). Thus, since TH-HZ mice presented lower NK activity than their WT littermates, at all ages of the study, it could confirm an altered neuroimmunoendocrine cross-talk in these partially *th* deficient female animals. Additional future analysis of males will clarify if the finding is sex-dependent as well as validating the TH-HZ mice as a model of premature aging.

6. Conflicts of interest

The authors have no conflicts of interest to declare.

7. Acknowledgments

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Tables

Table 1. Sensorimotor abilities in TH-HZ and WT mice at adult and old age.

	ADULT		OLD	
	WT	TH-HZ	WT	TH-HZ
Weight (g)	41±7	42±5	44±7	41±9
Visual placing reflex				
% Mice showing this response	100	100	100	100
Hindlimb extensor reflex				
% Mice showing this response	100	100	100	100
Wood rod test				
Motor coordination				
Latency to leave the starting segment (s)	6±3	16±7 ***	10±7	41±18 ***
Total number of crossing segments	3±1	5±2	4±1	4±1
Tightrope test				
Motor coordination				
Latency to leave the starting segment (s)	9±6	33±21 **	17±0 bb	24±23
Total number of crossing segments	3±1	3±1	2±1	2±1
Muscular vigor				
% Mice falling off the rope	25	20	88 bb	83 bb
Latency to fall (s)	25±9	15±4 **	16±3 b	5±4 *** bbb
Traction				
Maximum	83	70	48 b	50 b

Each value represents mean ± standard deviation of 10 values corresponding to that number of animals. ** $p<0.01$, *** $p<0.001$ with respect to WT group; b $p<0.05$, bb $p<0.01$, bbb $p<0.001$ with respect to value obtained for these animals at adult age of same genotype.

Table 2. Exploratory and anxiety-like behaviors in TH-HZ and WT mice at adult and old age.

	ADULT		OLD	
	WT	TH-HZ	WT	TH-HZ
Elevated Plus Maze				
Anxiety-like behavior				
Total number of entries in open arms	5±2	3±1 *	3±1 b	2±2
% Time in open arms	(see Fig 2A)			
Total number of entries in closed arms	9±3	11±1	10±3	11±3
% Time in closed arms	53±12	72±4 ***	64±15	71±6
% Time in central platform	33±9	22±1	25±5	25±5
Total number of grooming	3±1	12±2 ***	1±1	6±2 *** bbb
Holeboard test				
Non goal directed behavior				
Vertical activity				
Total number of rearing	(see Fig 2E)			
Time of rearing (s)	8±1	1±1 ***	4±1 bbb	0 ***
Horizontal activity				
Total locomotion	(se Fig. 2C)			
% inner locomotion	11±2	7±3	7±2 bbb	5±1
% external locomotion	48±4	69±5 ***	61±5 bbb	68±4
Goal directed behavior				
Total number of head-dipping	(see Fig 2F)			
Total time of head-dipping (s)	63±8	9±2 ***	25±5 bbb	12±4 ***
Self-grooming and -freezing behaviors				
Total number of grooming	(see Fig 2B)			
Time of grooming (s)	0	20±4 ***	7±4 bbb	15±4
Total number of freezing	0	8±2 ***	2±1 b	6±1
Time of freezing (s)	0	12±4 ***	2±1 b	7±2
T-maze test				
Horizontal activity				
Time for crossing the intersection of the maze (s)	6±2	12±2 ***	14±2 bbb	21±3 *** bbb
Time spent exploring the entire maze (s)	(see Fig 2D)			

Each value represents mean ± standard deviation of 10 values corresponding to that number of animals. * $p<0.05$, *** $p<0.001$ with respect to WT group; b $p<0.05$, bbb $p<0.001$ with respect to value obtained for these animals at adult age of same genotype.

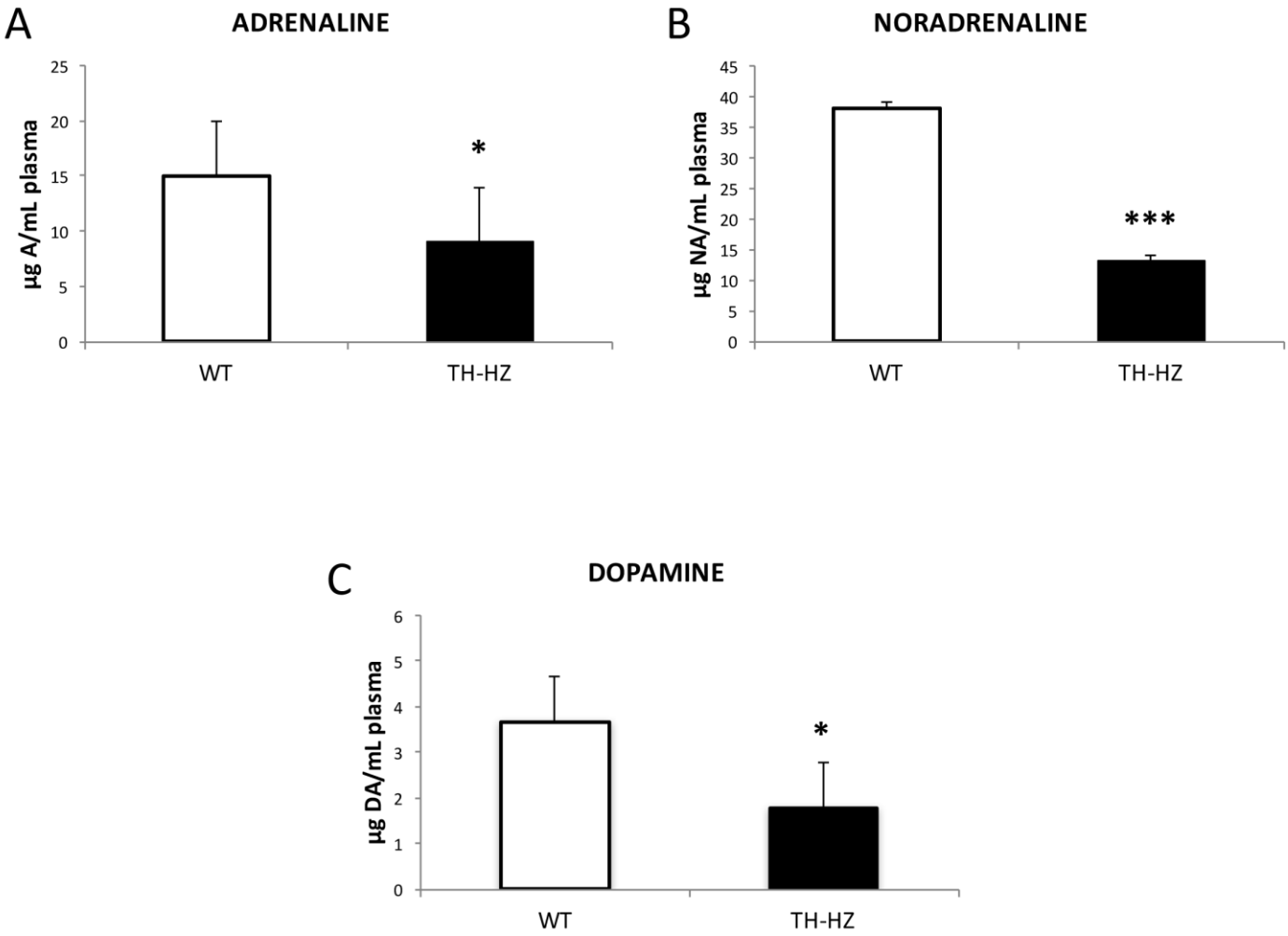
Table 3. Parameters of oxidative and inflammatory stress as well as resting lymphoproliferation of peritoneal leukocytes from TH-HZ and WT mice at 2±1 months, 4±1 months, 9±1 months, 13±1 months, and 20±1 months.

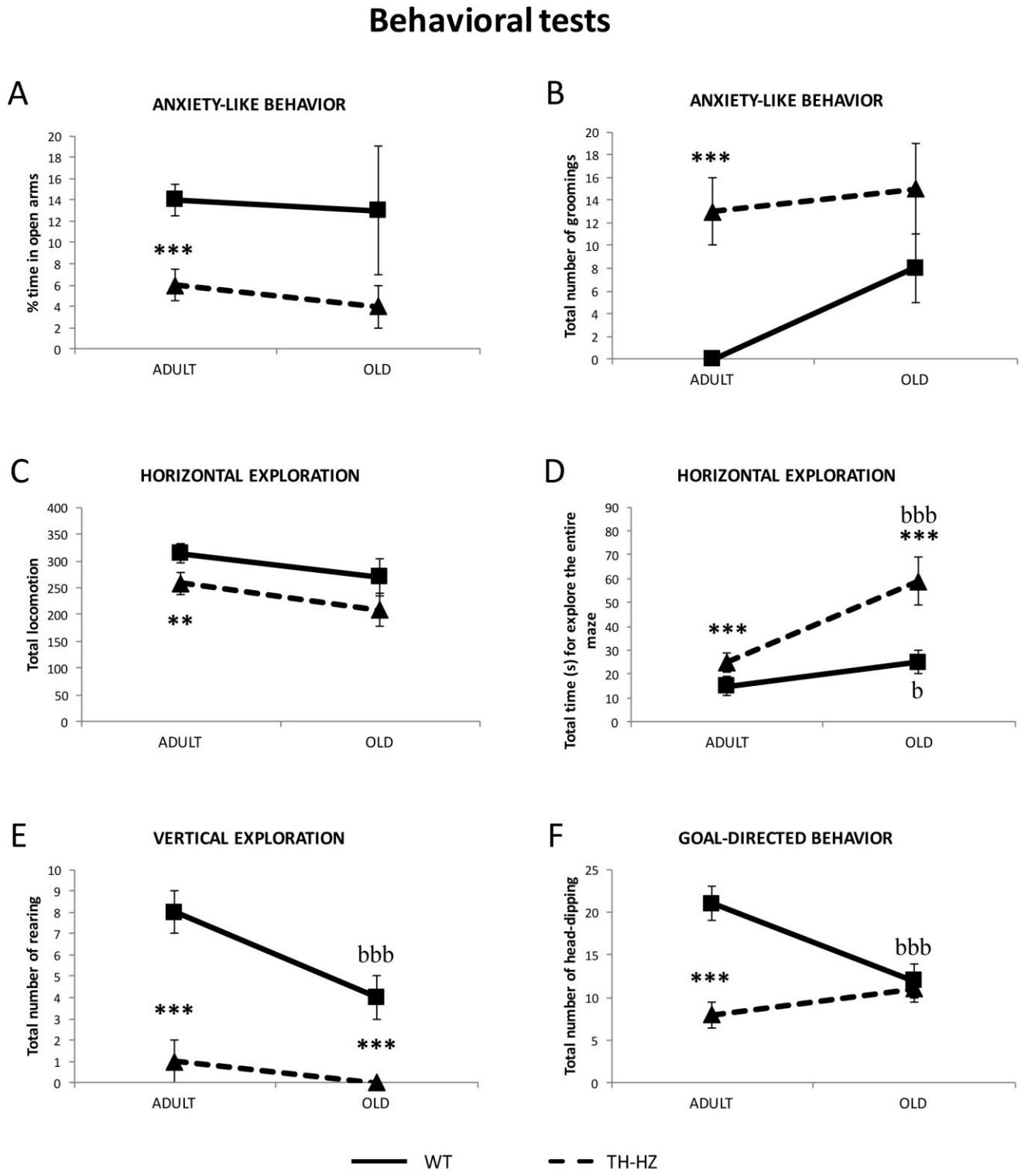
	2 months		4 months		9 months		13 months		20 months	
	WT	TH-HZ	WT	TH-HZ	WT	TH-HZ	WT	TH-HZ	WT	TH-HZ
Parameters of oxidative and inflammatory stress and resting lymphoproliferation										
Oxidative stress										
Antioxidants										
Catalase activity (UI CAT/10^6 leukocytes)	28±6 bbb	6±4 ***	12±2	8±3	11±5	6±1	10±6	5±4	9±3	6±2
Glutathione reductase activity (mU GR/10^6 leukocytes)	(see Fig. 4A)									
Glutathione peroxidase activity (mU GPx/10^6leukocytes)	122±45 bbb	245±60 *** bb	103±33 bbb	51±16 ** bbb	475±87	386±71	598±116	492±108	396±25	396±101
Reduced glutathione (nmol GSH/10^6 peritoneal leukocytes)	(see Fig. 4B)									
Oxidants										
Oxidized glutathione (nmol GSSG/10^6 peritoneal leukocytes)	2,31±0,23 bb	2,45±0,11 b	2,05±0,48	2,35±0,31	1,48±0,51	2,13±0,15	3,21±1,09 bb	4,90±1,32 bbb	4,87±0,37 bbb	5,63±0,13 * bbb
Xanthine oxidase activity (U XO/10^6 peritoneal leukocytes)	(see Fig. 4C)									
GSSG/GSH ratio	(see Fig. 4D)									
Inflammatory stress										
Cytokines levels in resting lymphoproliferation										
TNF-alpha (pg/ml)	(see Fig. 4E)									
IL-10 (pg/ml)	204±68	257±48	193±54	287±137	210±34	294±38 **	192±32	288±76	122±49 bb	114±93
TNF-alpha/IL-10 ratio	(see Fig. 4F)									
Resting lymphoproliferation (c.p.m.)	4016±757 bbb	5329±675 *	2851±678 b	4951±911 **	1476±382	4269±717 ***	2654±562 b	3286±731	3519±912 bbb	3450±717 b

Each value represents mean ± standard deviation of 10 values corresponding to that number of animals. * $p<0.05$, *** $p<0.001$ with respect to WT group; b $p<0.05$, bbb $p<0.001$ with respect to value obtained for these animals at adult age of same genotype.

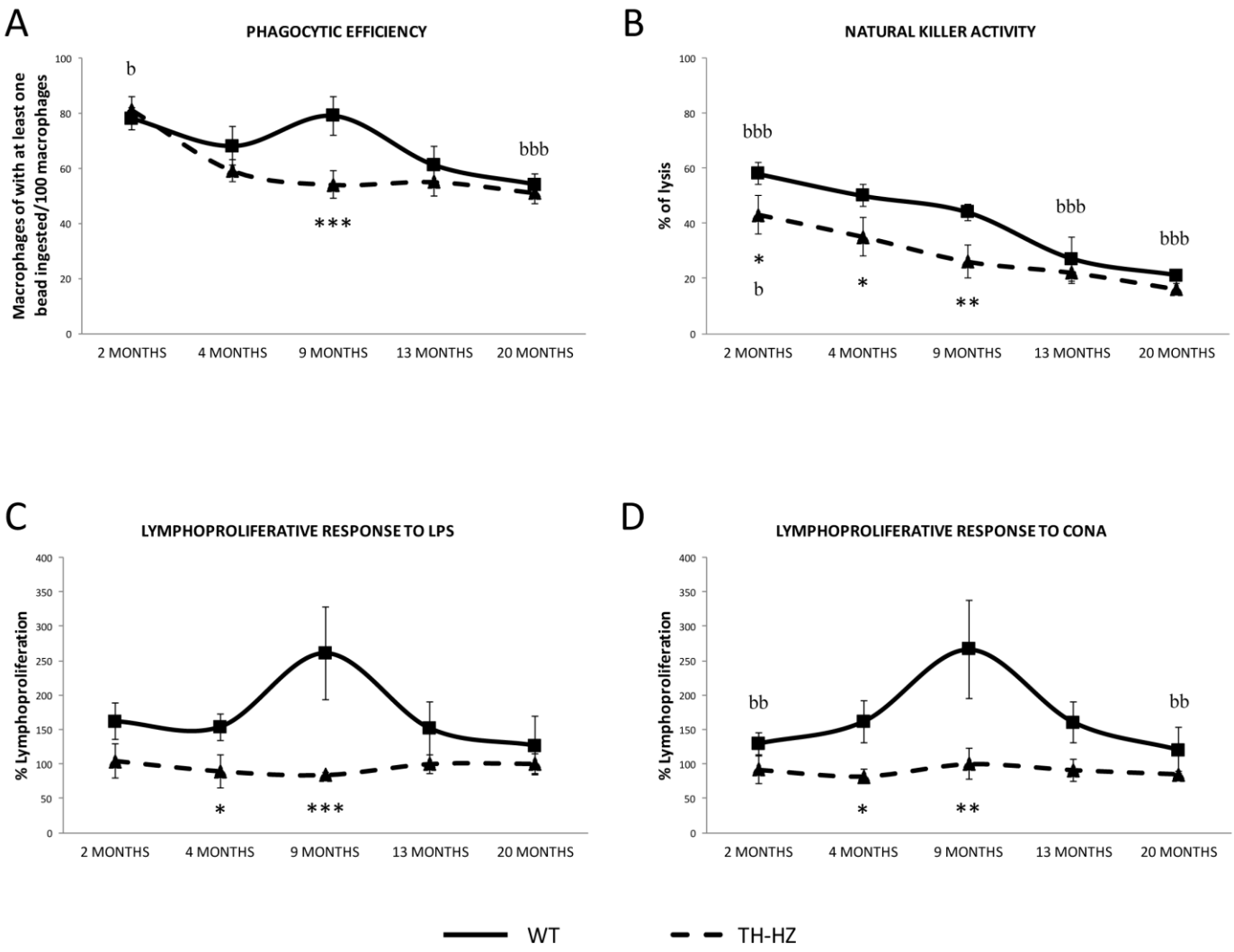
Highlights

- TH-HZ adult female mice exhibit behavioral impairment.
- TH-HZ adult female mice show premature immunosenescence.
- TH-HZ female mice present oxidative and inflammatory stress of the immune cells.
- TH-HZ female mice exhibit a significantly shorter mean lifespan.
- Tyrosine hydroxylase haploinsufficiency provokes premature aging.





Immune function



Parameters of oxidative and inflammatory stress