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RESEARCH ARTICLE

Sex-related differences in behavioural markers in adult mice for the prediction of lifespan

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Abstract Finding biomarkers to assess the rate of ageing and consequently, to forecast individual lifespan is a challenge in ageing research. We recently published a mathematical model for lifespan prediction in adult female mice using behavioural parameters such as internal locomotion and time spent in open arms in the hole board (HB) and elevated plus maze (EPM) tests, respectively. Nevertheless, it is still not known if these behavioural variables could be useful in forecasting lifespan in male mice. Therefore, two groups of ICR-CD1 mice, male and female were subjected to the EPM, HB and T-maze tests at the adult age. Mice were monitored until they died and individual lifespans were registered. In general, adult male mice showed more anxiety-like behaviours than females. The mathematical model previously developed in females was validated with the female cohort, but found to be suboptimal for lifespan prediction in males. Thus, a new model for male lifespan prediction was constructed including the behavioural variables that were predictive of lifespan in males: time in the central platform of the EPM, inner locomotion, number of groomings and number and duration of head-dippings in the HB. These results confirm that the higher the anxiety-like behaviour at the adult age, the shorter the lifespan.

Keywords Male and female mice behaviour · Lifespan prediction · Anxiety and exploratory behaviour · Multiple lineal regression

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Introduction

It is known that the rate of ageing and therefore, the expected lifespan differs between individuals with the same chronological age (Collier and Coleman 1991). Because of this, chronological age proves to be an unreliable tool in the estimation of the rate of ageing.

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This leads to the necessity of finding appropriate biomarkers that will allow us to better evaluate this rate and to forecast individual lifespan. Since life expectancy data are difficult to collect in humans due to their long lifespan, mice, which have a mean longevity of around two years, are more suitable for the study and validation of biomarkers for the prediction of lifespan.

However, the question that remains is, which set of parameters have the potential to be accurate for the prediction of lifespan? It is known that ageing is accompanied by the progressive deterioration of the three main homeostatic systems: the endocrine, immune and nervous systems, which lose their regulatory capacity resulting in an increase of morbidity and mortality (Chahal and Drake 2007; De la Fuente and Miquel 2009). Therefore, it can be hypothesized that the early emergence of age-related changes in these homeostatic systems would influence the final lifespan achieved.

In fact, parameters concerning the performance of the homeostatic systems have been suggested as markers of the rate of aging, both in humans and mice. Such parameters include blood hormone levels (Bae et al. 2008), lymphocyte subsets (Miller 2001) and immune function parameters (Martínez de Toda et al. 2016). In order to evaluate the performance of the homeostatic systems in mice, the one that can be easily assessed, by carrying out behavioural tests, is the functioning of the nervous system. These tests provide cheap, non-invasive and easy-to-access data (Gilad and Gilad 1995; Dellu et al. 1996).

Actually, some behavioural indices have been postulated as markers of premature ageing in rodents. This is the case of the fear of novelty in rats (*neophobia*) (Cavigelli and McClintock 2003) or anxiety/emotionality in mice measured in the T-maze (Viveros et al. 2001, 2007). Thus, groups of rodents with a higher fear of novelty or a higher anxiety at the adult age showed a shorter lifespan than those with lower emotionality. For its importance as a readout of the function of the nervous system, its proven relevance in ageing and its accessibility, in this study we focused on the behavioural performance of mice at the adult age. Two of the most commonly used tests to assess the anxiety state and exploratory capacities in rodents are the elevated plus maze (EPM) and the hole board (HB) tests, respectively (File and Day 1972; Walf and Frye 2007).

However, very few studies investigated the applicability of behavioural parameters to predict individual lifespans by the construction of mathematical models. In this regard, it was recently demonstrated by our research group that two behavioural indices, namely internal locomotion in the HB test and time spent in open arms in the EPM test, measured at the adult age in female ICR-CD1 mice, can be combined into a mathematical model to predict individual lifespan in female mice (Martínez de Toda et al. 2019). Nevertheless, it is still not known if the above mentioned indices could also be used for lifespan prediction in males, considering the behavioural differences that have previously been reported between male and female mice (Frick and Gresack 2003; Tanaka 2015; Yokota et al. 2017).

Therefore, male and female ICR-CD1 mice, were subjected to the elevated plus maze, the hole board and the T-maze tests at the adult age (40 weeks-old). Both groups were monitored individually throughout the ageing process until they died and individual lifespans were registered. This approach allowed us firstly to investigate the behavioural differences between male and female adult ICR-CD1 mice and secondly, to ascertain the applicability and reproducibility of the previously developed mathematical model for females in the new group of male and female mice, respectively. Finally, it allowed us to identify the strongest behavioural parameters for lifespan estimation in males by developing a mathematical model using multiple linear regression (MLR) with the behavioural indices obtained in adult male mice.

Methods

Experimental animals

Two groups of male (N = 43) and female (N = 45) outbred ICR-CD1 mice (*Mus musculus*) were obtained from Janvier Labs (Germany) at the age of 30 ± 4 weeks. They were housed in separate rooms, under standard conditions in 21.5 × 46.5 × 14.5 cm transparent policarbonate cages with a wire mesh top, between 4 and 6 mice per cage. Standard wood chip bedding was used, with a reversed day/night cycle of 12:12 h (lights off at 0800 h) with access to standard pelleted food (Envigo Teklad, USA) and tap water ad libitum. These mice were left to acclimatize to the

new housing conditions for 10 weeks and then subjected to the behavioural tests described below. Mice were subjected to each test only once at the adult age of 40 ± 4 weeks and were maintained in the same housing conditions until their natural deaths, which were registered (observed lifespan).

Anxiety and exploratory behavioural tests

All tests were performed in a different room as the housing room and after a period of acclimatization to the experimentation room of 1 h. The three tests were performed on individual mice in increasing order of potential stress produced in the animals in order to have the minimal possible impact in the following tests (i.e. T-maze first, then EPM and HB last) (Crawley 2008) and the ones done by Martínez de Toda et al. (2019) for maximum reproducibility. Mice were individually tested once per test, with an interval of one week between each test. All tests were performed between 0830 and 1130, so that behaviour was not affected by circadian rhythms and the apparatus was cleaned between mice with 70% ethanol and then water to remove possible olfactory cues from the previous mouse.

T-maze

The T-maze is a T-shaped structure with a vertical arm of 27 × 10 cm and a horizontal arm of 64 × 10 cm, which are bordered by 19 cm high black wooden walls. The base of the apparatus is made of 0.5 cm diameter metal rods perpendicular to the orientation of each arm, each being separated by a 0.5 cm gap. Mice were placed one by one in the lower part of the vertical arm facing the wall, under red ambient light with a 20 W dim white spotlight and were allowed to explore the maze. The point in time at which all four paws of the mouse crossed to the horizontal arm was recorded as a measure of the spontaneous exploratory capacity.

Elevated plus maze

The Elevated Plus-Maze (EPM) was acquired from Panlab (Spain) and consists of a cross shape composed of two opposed open arms (OA) and two opposed closed arms (CA), each of 30 × 5 cm black wood. The CAs are surrounded on three sides by 40 cm high black wooden walls meanwhile the OAs lacked walls. These arms intersect at a central platform (CP) that measures 5 × 5 cm (also in black). The set-up is elevated 40 cm from the floor. Mice were placed individually on the CP facing an OA and were left to explore freely for 5 min under red light with a 75 W bright white spotlight. To ensure minimum interference, the scientist left the experimentation room and the test was recorded on video and was later used to obtain the parameters of total time spent in OAs, total time spent in CAs, total time spent in the CP, as well as the number of entries into each type of section (OA, CA and CP), an entry being considered when all four paws were placed into the same section.

Hole board test

The Hole Board (HB) is an enclosed square-shaped black board (60 × 60 × 45 cm) divided into 36 squares (10 × 10 cm), with four circular holes (3.8 cm in diameter) in the corners of the four central squares, each containing a white plastic object to motivate inspection by the mice. The 20 squares adjacent to a wall segment were considered "external" and the rest "internal" squares. Mice were placed one by one in a corner facing the wall and left to freely explore for 5 min under red light with a 75 W bright white spotlight and was video recorded. The following parameters were analysed: horizontal exploration, measured by total locomotion (total number of squares crossed), internal and external locomotion as total number of squares crossed and as a percentage of the total locomotion. Moreover, vertical exploration, measured by the number and duration of positions in which the mouse raises its body more than 45° on its hind paws (rearings with or without support from walls) was also assessed. As a measure of "directed exploration" the number and duration of positions in which the mouse introduces its head into a hole (head-dippings) were recorded. The number and duration of positions in which the mouse grooms its face, body or both (groomings) were also quantified.

All measured variables are summarized in Table 1.

Table 1 Variables measured in each behavioural test

Test	Parameter	Description
T-maze	Time to cross the intersection	Time (s) needed for the mouse to cross the metal rod separating the vertical and the horizontal arms with its four paws
Elevated Plus Maze	Time spent in CP	Time (s) of permanence in the central platform under the four paws criteria
	Time spent in OA	Time (s) of permanence in either of the open arms under the four paws criteria
	Time spent in CA	Time (s) of permanence in either of the closed arms under the four paws criteria
	Number of entries into the CP	Number of crossings to the central platform from any other section
	Number of entries into OA	Number of crossings to open arms from the central platform
	Number of entries into CA	Number of crossings to closed arms from the central platform
Hole Board	Total locomotion	Total number of squares traversed through under the four paws criteria
	Inner locomotion	Number of squares traversed through in areas not directly adjacent to the wall
	Outer locomotion	Number of squares traversed through in areas directly adjacent to the wall
	Percentage of inner locomotion	Inner locomotion divided by total locomotion times 100
	Percentage of outer locomotion	Outer locomotion divided by total locomotion times 100
	Number of supported rearings	Number of positions in which the mouse stands on the rear paws lifting its body more than 45° from the ground supported against a wall
	Time in supported rearing position	Accumulated permanence (s) in supported rearing positions
	Number of unsupported rearings	Number of rearing positions without support from walls
	Time in unsupported rearing position	Accumulated permanence (s) in unsupported rearing positions
	Number of head-dippings	Number of positions in which the mouse inserts its head into a hole up to its ears
	Time in head-dipping position	Accumulated permanence (s) in head-dipping positions
	Number of groomings	Number of positions in which the mouse grooms itself
	Time in grooming position	Accumulated permanence (s) in grooming positions

CP central platform; *OA* open arms, *CA* closed arms

Statistical procedures

Behavioural comparison between males and females

The observed lifespans of male and female groups were recorded employing a Kaplan–Meier survival plot and were compared using a log rank test.

The normality of the behavioural performance of each sex was evaluated using a *Kolmogorov–Smirnov* test and the homoscedasticity using a *Levene* test (data not shown). Comparisons between the variables of each sex were carried out using a two-tailed *Student's t* test for unpaired samples with a minimum significance level of $p < 0.05$.

Validation and cross-sex confirmation of the previous female mathematical model

The cross-validations were evaluated using Pearson´s correlation coefficient applying the previous female two-parameter model (Eq. 1, as described in Martínez

de Toda et al. (2019) with the newly obtained male and female data.

$$\begin{aligned}\text{Estimated lifespan} = {} & 11.833 + 0.378 \\ & \times (\text{Internal locomotion}) \\ & + 0.202 \\ & \times (\text{Time spent in open arms}).\end{aligned} \quad (1)$$

New behavioural model construction for males and validation

The new mathematical model was generated using Multiple Lineal Regression (MLR) with SPSS 21 (IBM SPSS Statistics) with a forward step-wise method, in which the variable most useful for prediction is selected and the variables most fitting with the observed lifespan, considering those previously introduced, are successively selected with a threshold of $p < 0.05$. The observed lifespan was used as a dependent variable and the rest as independent variables.

This newly generated male model was validated as well by the Pearson´s correlation coefficient comparing the observed vs the estimated lifespan in the male group.

Results

Behavioural comparison between males and females

The results obtained in the different behavioural parameters analysed in adult male and female ICR-CD1 mice are shown in Figs. 1 and 2 as well as in Table 2.

Exploratory behaviour

Differences due to sex were observed with respect to the exploratory behaviour in both horizontal and vertical exploration in the HB (Fig. 1). Horizontal exploration was measured by the raw amount of square divisions traversed in the HB test, in which females performed a significantly higher locomotion in the inner ($p < 0.05$, Fig. 1a) but not in the outer regions of the board ($p = 0.206$, Fig. 1b), nor in the total count of traversed squares, than males (total locomotion, $p = 0.646$, Table 2). However, with respect to vertical exploration, represented by the number (Fig. 1c) and time (Fig. 1d) of unsupported rearing positions, male mice had a higher number and duration of rearing than females ($p < 0.001$ and $p < 0.05$, respectively). Nevertheless, no significant differences were found between male and female mice regarding goal-directed exploration, measured by the number and duration of head-dipping positions (data shown in Table 2).

Anxiety-like behaviour

Other variables that can be categorized as anxiety-like parameters are shown in Fig. 2. First, inner and outer locomotion were obtained as a percentage, dividing the number of internal or external crossed squares by the total squares crossed in the HB. It was observed that female mice had a higher percentage of inner locomotion and lower outer locomotion than males ($p < 0.001$, Fig. 2a, b, respectively). With respect to the number of grooming behaviours in the HB, these were more abundant in females compared to males ($p < 0.001$, Fig. 2c), but there were no statistically significant differences in total time performing grooming behaviours (data shown in Table 2). Regarding rearings in the HB, males spent more time than females in rearing positions supported against the wall ($p < 0.001$, Fig. 2d), but there were no statistically significant differences in the total number of supported rearings each sex performed (data shown in Table 2). In addition, male mice took significantly longer average times to reach the intersection between arms in the T-maze test than females ($p < 0.05$, Fig. 2e). And concerning the derived indices from the EPM, it was found that male mice spent more time in the CP of the maze than females ($p < 0.001$, Fig. 2f), whereas no statistically significant differences were observed regarding the time spent or the number of entries in open or closed arms (data shown in Table 2).

Mice survival

In this study, male mice lived between 48 and 115 weeks (mean ± standard deviation of 79 ± 15 weeks) and females between 41 and 132 weeks (78 ± 24 weeks). Kaplan–Meier survival curves showed no statistical difference between male

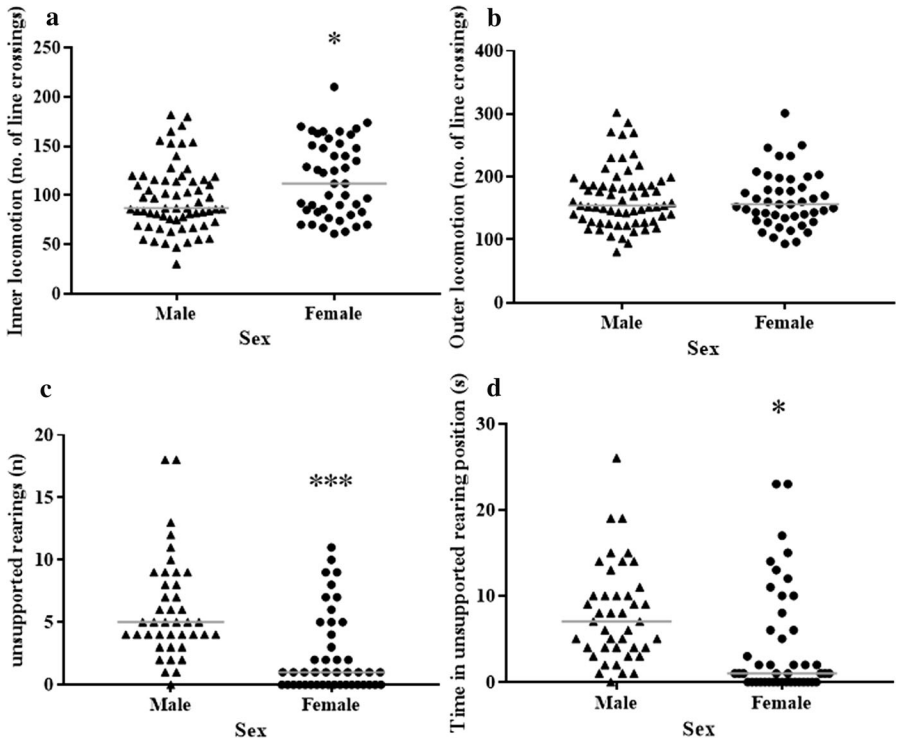

Fig. 1 Comparison of exploration-related parameters between adult male and female ICR-CD1 mice. **a** Number of inner squares and **b** Number of outer squares crossed in the HB test; **c** Number of unsupported rearings and **d** Time in unsupported rearing position in the HB test. Individual data for each mouse and the median of the values obtained for each parameter are depicted. $^{*}p < 0.05$ $^{***}p < 0.001$

and female survival ($\chi^2 = 0.199$, $p = 0.655$, data not shown).

Application of the model for female mice lifespan prediction previously developed to the new batches of both sexes

The previously published mathematical model for lifespan prediction in female mice (Martínez de Toda et al. 2019) was applied to the new two cohorts of male and female mice used in this study and represented in a scatter plot. This resulted in a *Pearson´s* correlation coefficient of 0.774 ($p < 0.001$) between predicted and observed lifespan for females and a *Pearson's* correlation coefficient of 0.421 ($p = 0.005$) for male mice (Fig. 3).

Generation of a male lifespan prediction model based on behavioural indices

To develop a mathematical model for the estimation of the lifespan of male mice based on their behavioural data gathered at the adult age, a Multiple Lineal Regression model (MLR) was used. The first and most predictive variable selected was the time spent in the CP of the EPM, which accounted for 43.3% of the observed variance in lifespan. With the introduction of the second variable (percentage of internal locomotion in the HB) the explained variance increased to 50.5%. The number of head-dippings was selected as the third variable and it rose to 56.6%. The fourth variable was the number of groomings performed in the HB, which increased the explained variance to 61.8%, and the fifth and final variable included (duration of head-dippings) resulted in an increase of the explained variance in individual lifespan of the model to 63.6%. These results are summarized in Table 3.

Therefore, the equation obtained for male lifespan prediction based on behavioural data is shown in Eq. 2.

$$\begin{aligned}\text{Estimated lifespan} = {} & 48.8 + 0.33 \\ & \times (\text{Time in central platform}) + 0.77 \\ & \times (\text{Percentage of internal locomotion}) - 1.52 \\ & \times (\text{Number of head} - \text{dippings}) - 3.42 \\ & \times (\text{Number of groomings}) + 0.15 \\ & \times (\text{Duration of head} - \text{dippings}).\end{aligned} \quad (2)$$

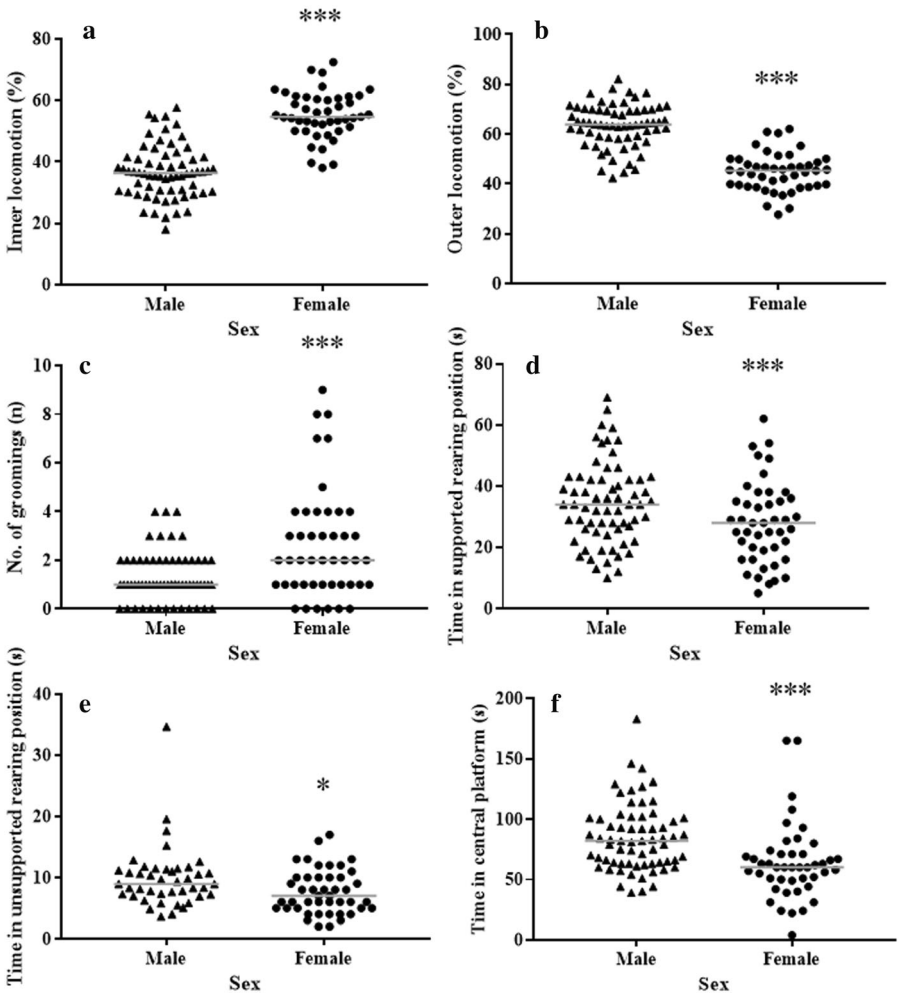

Fig. 2 Comparison of anxiety-related parameters between adult male and female ICR-CD1 mice. **a** Percentage of inner locomotion and **b** Percentage of outer locomotion in the HB. **c** Number of groomings in the HB. **d** Time in supported rearing positions in the HB. **e** Time needed to reach the intersection in the T-maze. **f** Time spent in CP in the EPM. Individual data for each mouse and the median of the values obtained for each parameter are depicted. ***$p < 0.001$; *$p < 0.05$

Table 2 Behavioural variables in which no statistically significant differences were found between adult male and female ICR-CD1 mice

Variable	Male		Female	
	Mean	SEM	Mean	SEM
Total locomotion	266	10	273	9
No. of supported rearing positions (n)	25.8	1.5	21.4	1.8
No. of head-dippings (n)	17.3	0.8	18.2	0.9
Time in head-dipping position (s)	50.4	3.5	56.6	4.8
Time to reach centre squares (s)	35.1	3.7	29.0	3.7
Time in grooming position (s)	6.0	0.9	7.0	0.9
Time in closed arms (s)	125.7	5.6	143.8	9.6
Time in open arms (s)	91.6	5.6	104.8	10.4
No. of closed-arm entries	11.4	0.5	10.5	0.6
No. of open-arm entries	8.7	0.7	7.7	0.7

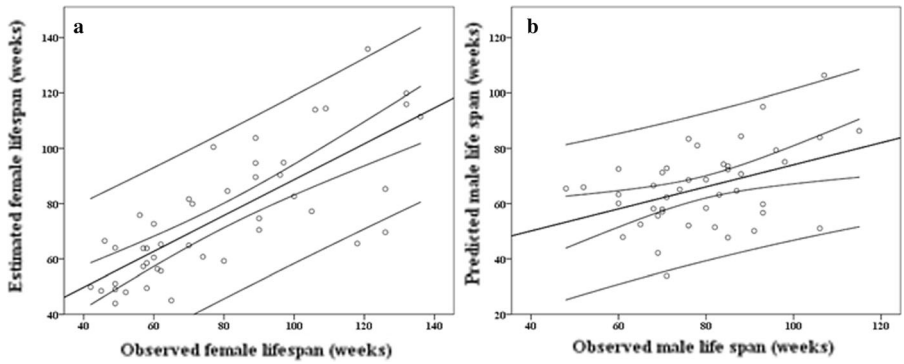

Fig. 3 Correlations between observed and predicted lifespans when applying the mathematical model previously developed with female mice, to the new cohorts of female (**a**) and male mice (**b**) of this study. The closest lines are the mean 95% confidence interval and the furthest lines correspond to the individual 95% confidence intervals. The application of the previous female mathematical model to the new female mice results in a *Pearson's* correlation coefficient of 0.774 with a p value lower than 0.001 and a *Pearson's* correlation coefficient of 0.421 with a p value of 0.005 for males in the new cohorts

Table 3 The generated lifespan prediction model in male mice

Steps	1 variable	2 variables	3 variables	4 variables	5 variables
R^2	44.7%	52.9%	59.7%	65.5%	67.9%
Adjusted R^2	43.3%	50.5%	56.6%	61.8%	63.6%
Constant ($\widehat{\beta_0}$)	42.8(6.6)	29.1(8.0)	37.9(8.3)	49.3(9.0)	48.8(8.8)
Time in central platform ($\widehat{\beta_1}$)	0.44(0.08)***	0.33(0.08)***	0.35(0.08)***	0.32(0.07)***	0.33(0.07)***
Internal locomotion (%) ($\widehat{\beta_2}$)	–	0.64(0.24)*	0.74(0.23)**	0.75(0.22)***	0.77(0.21)***
Number of head-dippings ($\widehat{\beta_3}$)	–	–	− 0.81(0.32)*	− 1.00(0.31)**	− 1.52(0.43)***
Number of groomings ($\widehat{\beta_4}$)	–	–	–	− 3.53(1.40)*	− 3.42(1.37)*
Duration of head-dippings ($\widehat{\beta_5}$)	–	–	–	–	0.15(0.09)

Each column depicts each additional variable introduced in the model and the rows contain the R^2 and adjusted R^2 values for each of the sets of variables considered and the coefficients for each variable with the standard mean error in brackets

*$p < 0.05$ **$p < 0.005$ ***$p < 0.001$

Validation of the male-model

To test whether the newly generated male lifespan prediction model correlated better with the observed lifespan of the male mice, the observed and predicted values of the lifespan of each mouse were represented in a scatter plot (Fig. 4). The correlation between the model and male data was assessed by *Pearson's* correlation coefficient with a value of 0.824 and a significance of $p < 0.001$. However, when this newly generated mathematical model was applied to female mice only a non-significant ($p = 0.238$) *Pearson*'s correlation coefficient of 0.180 was obtained.

Discussion

To our knowledge, this study represents the first attempt at forecasting the individual lifespan of male mice at the adult age using easily accessible, non-invasive procedures, such as behavioural performance. The importance of knowing the approximate lifespan of a mouse at the adult age based on behavioural parameters is threefold. Firstly, this would enable the testing of the effects that a given treatment, or a lifestyle factor has on the lifespan of each individual mouse, without having to wait until the natural death of the animal. Secondly, the pre-estimation of the expected lifespan of individual mice

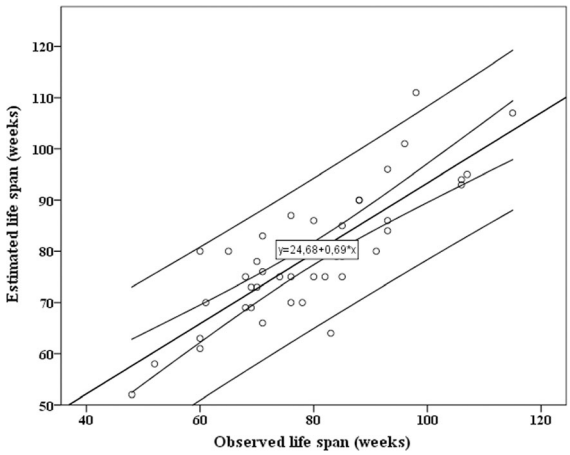

Fig. 4 Correlation between observed and estimated lifespan calculated with the newly generated five-variable model in the same cohort of male mice. The central line and equation represent the regression equation, the next closest lines are the mean 95% confidence intervals and the widest lines correspond to the individual 95% confidence intervals. The P*earson's* correlation coefficient value was 0.824 with a significance of $p < 0.001$

would allow us to homogenize control and experimental groups and therefore would diminish the heterogeneity of data. Thus, this would help to reduce the number of animals needed for an effective experiment. And thirdly, the identification of these predictive parameters could be of assistance in finding out the underlying mechanisms between a specific variable and longevity in males.

There have been previous attempts at predicting remaining lifespan in male mice based on behavioural and physiological parameters. However, these have been carried out in old or about-to-die mice (Ingram et al. 1982; Ray et al. 2010; Fahlström et al. 2012). More recently, some lifespan prediction models based on frailty characteristics have also been developed (Kane et al. 2019). Such lifespan prediction model reaches a slightly lower explained variance than the one described in this study but employs measurements dependant on varying degrees of technical equipment, personal expertise and possibly experimenter influence or bias. Additionally, most of the studies were performed using inbred mice, which tend to be more phenotypically homogeneous and would result in a more reproducible outcome (Phelan 1992). However, that homogeneity would limit the conclusions about the general ageing process in a more genetically diverse context, such as in humans. In the present study, we have used mice of the outbred ICR-CD1 strain and lifespan prediction has been carried out at the adult age. The earlier the lifespan prediction, the longer possibility of intervention, which could, in fact, show more beneficial effects. Supporting this idea, it was found that an environmental enrichment intervention in adult mice resulted in an increased lifespan, whereas the same strategy applied in old age was unable to increase longevity (Arranz et al. 2010). In addition, physical training in young mice was reported to have life-long benefits, even if the training was discontinued later on (Warden et al. 2007).

The differences between male and female have been always apparent but, only recently a consciousness has arisen for clinical and preclinical trials to be made in both sexes (Clayton and Collins 2014). Moreover, changes in yearly cycles (circannual rhythms) can have an important effect in many biological performances (Mate et al. 2014), These facts underscore the importance of carrying out these kinds of tests in both sexes and during a similar time of the year.

Previously published studies investigating behavioural differences between males and females in other strains, have shown conflicting results. For instance, some authors report females spending more time in the open arms (OA) of the EPM than males (Frick et al. 2000; Caldarone et al. 2008; Fahlström et al. 2011, 2012). Other authors describe less time spent in OA by females (Frick et al. 2000). Whilst some other studies found no substantial differences between the sexes (Fahlström et al. 2011, 2012). Thus, we decided to first investigate the differences between male and female mice subjected to several behavioural tests, namely the EPM, the HB and the T maze. The addition of the T maze from the previous study by Martinez de Toda et al. (2019) is justified as it was described as being useful for the detection of anxiety-like behaviours which ultimately influence the rate of ageing and lifespan (Viveros et al. 2001).

With respect to exploratory behaviours, the results demonstrate that males performed better in vertical exploration than females (measured as number and duration of unsupported rearings), which in turn, performed more horizontal explorations (measured as total inner locomotion in the HB test), whereas there were no differences regarding the outer and the total locomotion between sexes. Different studies reported conflicting results in horizontal exploration not only

between the sexes, but also between strains (Gioiosa et al. 2007; Arndt et al. 2009). However, regarding the vertical component of exploration, our results agree with what has been reported for this, and other strains of mice (An et al. 2011). Indeed, this together with the fact that males tend to show greater neuromuscular vigour and coordination than females (Viveros et al. 2001) could lead one to think that both sexes show a similar drive towards exploration within the scope of their capabilities, males performing more rearings to explore distant spaces and females engaging in closer inner exploration.

In regard to anxiety-like behaviour, it was found that female mice spend a higher percentage of time exploring the inner region of the HB test than males, while males spend a higher percentage of time exploring the outer region of the HB test than females. Because mice have a natural aversion to the brightly lit centre of an open field, the higher the exploration of the inner space in the hole board test, the less the anxiety-like behaviour (Kassed and Herkenham 2004). This suggests that males are more anxious than females. However, when looking at the number of groomings performed by each sex, it was found that females performed more grooming behaviours than males. This is striking as it has been previously reported that increased self-grooming correlates with an enhanced anxiety-like state (D'Amato and Pavone 1992; Kalueff and Tuohimaa 2005). However, other studies have reported for this and other strains, that the basal performance of this parameter is not comparable between males and females. Thus, conclusions about anxiety levels between sexes may not be made regarding groomings (Zhan et al. 2015; Amodeo et al. 2019).

Whereas unsupported rearings are considered an exploratory behaviour, supported rearings against the wall can be considered as an attempt by the mouse to escape a stressful situation and therefore they can be considered as an anxiety-like behaviour (Lever et al. 2006). Thus, the fact that male mice perform higher supported rearings than females reinforces the idea of males being more anxious.

Regarding the T-maze test, it has been postulated that the longer the time that a mouse needs to cross the intersection of the T, the higher the anxiety-like behaviour (Guayerbas et al. 2000). This is due to the fact that when anxious mice are placed in a new environment, they normally show freezing behaviour which makes them need more time to cross the intersection. Again, male mice took a longer time to cross the intersection of the T-maze than females.

In the EPM test, the time spent in closed arms is a classic indicator of anxious behaviour, given that mice feel safer in these arms protected by walls (Rodgers and Dalvi 1997). Nevertheless, we observed no differences with respect to this parameter between male and female mice. However, it was found that male mice spent more time than females in the CP of the EPM test. The CP can be considered a protected space (Rodgers and Johnson 1995), due to the partial lack of surrounding walls, but with easy access to enclosed areas. It is regarded as a readout of the decision making and waiting capacities in rats, although significantly affected by and related to the basal anxiety of the lab animals (Cruz et al. 1994; Clément et al. 2007; Casarrubea et al. 2015). Thus, it seems that males need more time than females in terms of decision making.

The next aim of our study was to investigate whether the previously developed mathematical model for lifespan prediction in female mice (Martínez de Toda et al. 2019) would be applicable to male mice. The behavioural indices that the female mathematical model included were the time spent in open arms in the EPM test and the total inner locomotion in the HB test, both variables with a positive constant, meaning the higher these parameters the longer the predicted individual lifespan. When applied to the new data gathered from male mice, it was found to be useful for prediction ($p = 0.005$) although not as accurate in males as in females (Pearson´s correlation coefficient 0.421 and 0.774, respectively). Nevertheless, this result demonstrates that the lower the anxiety-like behaviour a male mouse shows at the adult age, the longer it lives, as was previously demonstrated for female mice.

Still, we decided to use all the behavioural variables recorded at the adult age in male mice and through multiple linear regression developed a new model for males to identify if there were any other behavioural indices more related to final achieved lifespan in males. The selected variables were (in order of robustness into the mathematical model): time spent in the CP, percentage of inner locomotion, number of head-dippings, number of groomings and duration of head-dippings. The only variables that negatively

affected life expectancy were number of head-dippings and number of groomings.

As previously mentioned, the time spent in the CP can be regarded as an indication of the decision making and waiting capacities (Cruz et al. 1994; Casarrubea et al. 2015). Thus, it seems that the longer the time to think about and decide the next section to visit, the longer the lifespan in male mice. However, although it is an intriguing fact, the reasons behind this phenomenon are beyond the scope of this study. The next variable selected, percentage of inner locomotion, agrees with the previous study developed in female mice (Martínez de Toda et al. 2019). However, regarding head-dipping behaviours, the variables obtained seem controversial. On the one hand, the higher the number of head-dippings a mouse performs the shorter it lives but, on the other hand, the higher the time spent in head-dipping behaviour, the longer it lives. This may reflect that a greater commitment to exploration of a hole instead of short and repetitive glances could be the indication of a healthy nervous system. This is also hinted at by a study by Magaji et al., in which resveratrol treatment, one of the most widely accepted drugs to extend lifespan (Sinclair 2005), caused a reduction in the number of head-dipping behaviours in the same strain of mice (Magaji et al. 2017). This agrees with our result that fewer head-dippings correlate with longer lifespans. However, since resveratrol can have such pleiotropic effects, it is still too early to reach a conclusion about the causality of this behaviour in the context of organism longevity. Finally, the obtained model suggests that the higher the number of grooming behaviours, the shorter the lifespan in male mice, reinforcing the idea that grooming behaviour correlates with an enhanced anxiety-like state (D'Amato and Pavone 1992; Kalueff and Tuohimaa 2005).

To sum up, the variables that related most strongly with the age of natural death were anxiety-like behaviours, as the percentage of internal locomotion in the HB, time spent in CP of the EPM and number of groomings in the HB, demonstrating that the lower the anxiety-like behaviour a male mouse shows at the adult age, the longer it lives, as was previously shown for female mice (Viveros et al. 2001; Martínez de Toda et al. 2019).

Nevertheless, the new mathematical model developed for lifespan prediction in male mice was shown to not be useful for females. The discrepancies between male and female models remark the differences between sexes not only in the behavioural performance, but also in the sex-dependent role each behavioural variable play in the development of ageing itself.

One of the reasons male lifespan prediction needed more variables and resulted in a lower explained variance than in females, may be attributed to the natural aggressiveness of males when compared to females. As recently described by Razzoli et al., dominant-subordinate status among male mice can have important effects on age-related damage accumulation, such as increased senescence markers, metabolic and cardiovascular disorders and generally lower life expectancies (Razzoli et al. 2018). Even if partially mitigated by using littermates cohabitating in the same cage since birth, this aggressive behaviour could lead to wounds and infections with unforeseeable or unpreventable effects that could alter individual observed lifespans and therefore, compromise the predictive capacity of any model.

As limitations to this research, future studies with additional cohorts of male mice are needed to perform the cross-validation of the proposed lifespan prediction model. In addition, although previous studies have demonstrated that mice with anxiety have higher oxidative stress and worse immune functionality (Viveros et al. 2001, 2007; Masood et al. 2008), to further confirm the reliability of the results, information on the correlation of the selected behavioural variables with recognized markers of aging, such as those of oxidative stress or immunosenescence, will be required.

Nevertheless, the results of the present study show that first, male ICR-CD1 mice at the adult age display a different behaviour when compared to their age-matched female counterparts. Thus, males show symptoms of an increased anxiety-like state as seen in higher parameters of percentage of outer locomotion and time in supported rearing position in the HB, as well as time to reach the intersection in the T maze and time spent in the CP of the EPM, compared to females, although no difference in time spent in OA was seen. Secondly, the previously published lifespan prediction model based on behavioural data at the adult age in female mice was shown to have a significant correlation with observed lifespan in adult male mice, although with a lower accuracy than that observed in females, and conversely, the newly

generated 'male' lifespan prediction model was unsuccessful at predicting female lifespan, highlighting the importance of using both sexes in ageing studies. In addition, it was found that a different set of behavioural indices, such as time spent in the CP of the EPM, percentage of locomotion in the inner region of the HB, number of groomings and number of and duration of head-dippings in the HB were best for male lifespan prediction. The fact that time in CP of the EPM, rather than in OA, is more predictive of longevity in males may point towards different molecular mechanisms involved in dealing with decision making and anxiety in males and females.

Although further studies are needed to understand how behaviour affects lifespan in a sex-dependent manner, this study could potentially become a useful time-saving tool in longitudinal mice studies conducted on this strain and may help to decode the intrinsic pathways regulating the decline experienced during the physiological ageing process.

Author contributions HK and IMT performed the material collection, analysed and interpreted the generated data and drafted the manuscript. LSM gave insight and supervised the mathematical model construction, and revised the manuscript. MDF conceived and design this study, funded the project and critically supervised the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest Authors report no conflict of interest.

Ethical approval All procedures were approved by the Animal Experimentation Committee of the Universidad Complutense de Madrid.

Informed consent All authors have given explicit consent for publication of the current manuscript.

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