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Title The effects of aerobic exercise on depression-like, anxiety-like, and cognition-like behaviours over the healthy adult lifespan of C57BL/6 mice.

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Highlights for "The effects of lifelong aerobic exercise on depression-like, anxiety-like, and cognition-like behaviours over the healthy adult lifespan of C57BL/6 mice."

- Lifetime exercise can reduce overt anxiety in healthy ageing mice
- However lifetime exercise may increase neurogenesis-associated anxiety
- Exercise related freezing extended spatial learning latencies in young female mice
- Cognition in healthy ageing is both enhanced and impaired by lifelong exercise
- During healthy ageing, lifelong exercise did not impact notably on depression

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Abstract

Preclinical studies have demonstrated exercise improves various types of behaviours such as anxiety-like, depression-like, and cognition-like behaviours. However, these findings were largely conducted in studies utilising short-term exercise protocols, and the effects of lifetime exercise on these behaviours remain unknown. This study investigates the behavioural effects of lifetime exercise in normal healthy aging C57BL/6 mice over the adult lifespan. 12 week-old C57BL/6 mice were randomly assigned to voluntary wheel running or non-exercise (control) groups. Exercise commenced at aged 3 months and behaviours were assessed in young adult (Y), early middle age (M), and old (O) mice (n=11-17/group). The open field and elevated zero maze examined anxiety-like behaviours, depression-like behaviours were quantified with the forced swim test, and the Y maze and Barnes maze investigated cognition-like behaviours. The effects of lifetime exercise were not simply an extension of the effects of chronic exercise on anxiety-like, depression-like, and cognition-like behaviours. Exercise tended to reduce overt anxiety-like behaviours with ageing, and improved recognition memory and spatial learning in M mice as was expected. However, exercise also increased anxiety behaviours including greater freezing behaviour that extended spatial learning latencies in Y female mice in particular, while reduced distances travelled contributed to longer spatial memory and cognitive flexibility latencies in Y and O mice. Lifetime exercise may increase neurogenesis-associated anxiety. This could be an evolutionary conserved adaptation that nevertheless has adverse impacts on cognition-like function, with particularly pronounced effects in Y female mice with intact sex hormones. These issues require careful investigation in future rodent studies.

Abbreviations:
OF = open field;
EZM = elevated zero maze;
EPM = elevated plus maze;
FST = forced swim test;
BM = Barnes maze

Keywords Aging; Anxiety; Cognition; Depression; Exercise

1. Introduction

Depression, anxiety, and cognitive impairment are some of the most prevalent neuropsychiatric conditions currently requiring therapeutic solutions. World Health Organisation reports indicate that depression is currently the leading cause of disability worldwide [1]. The prevalence of depression is highest in young adulthood with reductions in middle age and increases in older age, and is twice as common in women [2, 3]. However there is considerable overlap between anxiety, depression, and cognitive impairment, and around 90% of patients with depression experience comorbid anxiety [4]. Depression also shows features of cognitive impairment with impairments to spatial and verbal memory and processing speed [5], and interestingly, recent work has found that mild cognitive impairment also increases the risk for depression and anxiety [6]. Furthermore, although it has been widely suggested that depression may increase the risk for dementia [7], current work shows this may not be the case [8], although depression in older individuals (aged over 65) may contribute to cognitive impairment [9]. Considered together, these findings are suggestive

of common pathways for these conditions, and this suggests that therapies that can treat all these conditions are preferable to those that address only one condition or another.

One therapy with potential for the prevention of depression, anxiety and cognitive impairment with aging is physical exercise. Rodent studies have shown chronic voluntary wheel running can be effective for improving depression-like, anxiety-like, and cognition-like behaviours [10-12]. For instance, adult mice that had *ad libitum* access to running wheels for three or four weeks have showed reductions in immobility time in the forced swim test (FST) [10, 13]. Similarly, studies examining 2 to 4 weeks of running in adult male C57BL/6 (wild type, WT) mice found exercise increased centre crossings and time spent in the centre of the open field [10, 14] suggesting chronic running reduces anxiety-like behaviours in adult male mice. Exercise induced changes in cognition-like functioning are consistent with the benefits of exercise for depression-like and anxiety-like behaviours. Improvements in latencies to locate the platform in the Morris water maze have been found in adult mice and rats following 4 weeks of running [15, 16]. Moreover, the beneficial effects of exercise for anxiety-like and cognition-like behaviours are also evident in aging animals [17]. Evidence of anxiolytic effects of exercise have been found in 18 month old animals, with increases in the time spent in the central zone, and reductions in time spent in the closed arms of the elevated plus maze (EPM) [17]. In addition, chronic running improved cognition-like behaviours as was evident in a greater number of correct entries in the radial maze in aging animals [17].

Chronic wheel running therefore appears to reduce anxiety-like and depression-like behaviours, and improve aspects of cognition-like behaviours in adult mice and rats, and these changes are also apparent in older animals. Notwithstanding this, there is limited work investigating the effects of chronic exercise on depression-like behaviours during normal healthy aging and in older robust and healthy animals. Nevertheless, considered together, these findings suggest that longer term exercise over the lifespan or active aging may have greater anxiolytic and pro-cognitive effects, and may reduce depression-like behaviours occurring with aging, and may have potential for more extensive changes in these behaviours. However, the majority of studies investigating the effects of chronic voluntary running on these behaviours of interest have been conducted with adult animals [10, 13-15, 18, 19] and this raises questions about the effects of lifelong exercise on behaviours with healthy aging animals. We hypothesise that long term exercise is associated with favourable behaviours compared to healthy age matched control non-exercise mice. This study therefore aims to investigate the effects of lifelong voluntary wheel running on ageing-associated alterations in anxiety-like, depression-like, and cognition-like behaviours in normal healthy aging male and female C57BL/6 mice over the adult lifespan from young adulthood, middle age, to older animals.

2. Methods

2.1 Animals

Eight week old C57BL/6 (WT) mice (n = 80, 38 males and 42 females) were purchased from the University of Adelaide. We chose C57BL/6 mice because they have shown the behavioural responses to exercise of interest [10, 20, 21]. Animals were first housed in IVC cages approximately 3-6 per cage and given at least 1 week to acclimatise to the facility prior to the commencement of the study. Mice were given *ad libitum* access to standard laboratory chow and water. Environmental conditions were maintained at 21±1 °C with a 12 hour light/dark cycle (lights on 7:00-21:00). This experiment was conducted and reported in accordance with the ARRIVE guidelines [22] for reporting in vivo experiments, and the University of Adelaide Animal Ethics Committee approved this study (M216-12). Animal husbandry and welfare was conducted according to ethical and animal facility guidelines, and the numbers of animals used and their suffering were minimised wherever possible.

2.2 Experimental design

At 12 weeks of age approximately equal numbers of healthy male and female mice (total n = 80) were block randomised in pairs for treatment allocation according to three time-points of behavioural evaluation (a) young adulthood; b) middle age; c) older middle age). These three cohorts remained independent throughout the study. All mice were transferred to open top caging and housed in pairs the duration of the study (unless fighting in males necessitated separation). Control mice (n = 38) were housed in standard open top housing (48.5cm x 15.5cm x 12cm) and received no exercise running wheel intervention. Exercise mice (n = 42) were housed in open top plexiglass cages (37cm x 20.5cm x 13.5cm) to accommodate the running wheel (12cm x 5.5cm). Wheel revolutions were manually recorded weekly from a digital counter. Exercise mice had continuous access to running wheels until different stages of adulthood: young adulthood [23], middle age [24], or older middle age [25] (Table 1). Behavioural testing was then conducted for four weeks (Table 1).

Table 1. Exercise durations for aging C57BL/6 mice.

Group	Start of exercise	Behavioural testing	No. of mice	Control (male: female)	Exercise (male: female)
Y	3 months	4 months	22	11 (6: 5)	11 (5: 6)
M	3 months	9 months	27	13 (6: 7)	14 (9: 7)
O	3 months	14 months	31	14 (7: 6)	17 (9: 12)

2.3 Behavioural analyses

Behavioural testing was conducted over 4 weeks. Tests were conducted in the light phase of the cycle and completed in order of the least to most stressful. To minimise the possible stressful effects of prior testing there was a minimum of one day between tests [26]. All testing was conducted utilising ANYmaze video tracking software (Stoelting Company (USA)). Tests were conducted in the following order 1) Home cage; 2) Open field; 3) Elevated zero maze; 4) Y maze; 5) Barnes maze; 6) Forced swim test. Testing areas were thoroughly cleaned between tests with F10SC Veterinary Disinfectant to remove any olfactory traces.

2.3.1 Locomotion

Baseline locomotion under non-stressful conditions was measured in the home cage with 2 day old bedding. The distance travelled was quantified for 5 minutes according to established protocols [27].

2.3.2 Open field test

We also quantified baseline locomotion and anxiety-like behaviours in more stressful conditions of the open field. Mice were placed in the North-West corner of a well-lit 40 x 40cm plexiglass box. The measures recorded included the distance travelled and time spent in the inner and outer zones of the field, and these were quantified for 5 minutes in line with published protocols [28]

2.2.3 Elevated zero maze

Anxiety-like and exploratory behaviours were also investigated using the elevated zero maze (EZM). The EZM is a circular elevated platform (40cm high, a diameter of 50cm, and 5cm wide) with four quadrants. Two closed quadrants have inner and outer walls 27cm high while the open quadrants have none. Mice were placed on the centre of the southern open quadrant to enable exploration. We recorded for 5 minutes to quantify head dipping behaviours and times spent in the open and closed quadrants.

2.3.4 Y maze

The Y maze was utilised to measure hippocampal dependent spatial recognition memory. The Y maze has 3 arms shaped as a 'Y' (35cm long, 5cm wide, and 10cm high) that are at 120° angle to one another [29] and is undertaken in 2 phases.

Phase 1

Mice were placed in the lower (southern arm) and allowed 10 minutes of exploration with one of the lateral arms closed.

Phase 2

Mice were again placed into the southern arm of the maze thirty minutes following phase 1. In phase two, the mazes' three arms are open for exploration for 5 minutes. Healthy mice with intact hippocampal learning and memory have a preference for the exploration of a novel environment [30]. However if mice have memory impairments they will not recognise the novel arm resulting in a greater amount of time being spent in the familiar arm of the maze.

2.3.5 Barnes maze

We utilised the Barnes maze to examine spatial learning and memory, and cognitive flexibility. The Barnes maze is a well-lit round table 91cm in diameter with 19 false escape boxes and 1 genuine escape box.

Training days, days 1 -4

The latency to locate the escape box over four days of training was used as a measure of spatial learning. On each of the four training days mice were placed in the centre of the table under a removable chamber. Mice were given 3 minutes to learn the location of the escape box. Mice that failed to learn the box's location were guided to its location where they resided for 1 minute. Animals were given 3 trials per day on each of the 4 days of training.

Probe trials

On day 5, the table was rotated clockwise 90 degrees. Mice were again placed on the centre of the table, and given 3 minutes to locate the new location of the escape box. Latencies to locate the escape box on day 5 were measures of spatial memory, and the latency to identify the new escape

box location were taken as a measures of cognitive flexibility because it constituted the time taken to change focus from the old location of the box, and identify its new location.

2.3.6 Forced swim test

Depression-like behaviour was quantified with the forced swim test. We quantified anhedonia type behaviour using immobility time as a measure of despair-like behaviour [31]. Immobility was defined as floating with or without the small movements that contribute to maintaining equilibrium, but that do not contribute to forward movement as in swimming or climbing.

2.3.7 Statistical methods

Primary outcomes were behaviours involved in the depression phenotype and included anxiety-like, depression-like, and cognition-like behaviours. The Shapiro-Wilk test for normality was used. To accommodate and analyse exercise distances travelled from groups of difference sizes (Table 1), analyses of mean daily distances travelled was conducted using linear mixed-effects modelling clustering on mouse for repeated measures, and utilising an AR(1) convergence structure for best model fit. The assumptions of normal distribution of residuals and homoscedasticity were confirmed by visual inspection of scatterplots and histograms of the residuals and predicted values. One model was fit for the mean daily distances travelled over the first eight weeks, and another for distances travelled from one to six months. Two-way ANOVA with Sidak's post hoc test for multiple comparisons were conducted for analyses of i) commencement and completion animal weights (control vs. exercise; male vs. female); ii) Barnes maze data between control and exercise groups and within groups over the 4 training days, and iii) to investigate differences between male and female animals in control and exercise groups in all behavioural tests. All other tests comparing control and exercise groups within each age cohort including differences between male and female mice were analysed by unpaired student's t tests for normally distributed data, and Mann-Whitney tests for data that was not normally distributed. Linear mixed-effects models were performed in SPSS (version 24), and the analyses of all other data were performed in GraphPad Prism (6 version 008, and version 7) and the data presented are mean ± SE.

3. **Results**

3.1 Animals

T-test analyses of animal body weights in Y, M, and O mice at the start and end of the experiment showed all groups maintained healthy body weights over the experiment. Female M and O control mice weighed significantly less than male M and O control mice at the start of the experiment ($p = 0.006$ and $p = 0.01$), whilst Y and M female exercise mice weighed less than Y and M exercise male mice at the experiment start ($p = 0.001$ and $p = 0.005$). At the end of the experiment, only M control and exercise female mice weighed less than their male counterparts ($p = 0.03$ and $p = 0.01$).Table 2. Mouse body weights at the start and end of the experiment.

Table 2. Mouse body weights at the start and end of the experiment.

Group	Start of experiment				End of experiment			
	Control		Exercise		Control		Exercise	
	m	f	m	f	m	f	m	f

Y	22.80 (±0.91)	20.40 (±0.24)	24.50 (±1.23)	19.6** (±0.61)	23.40 (± 1.32)	22.0 (±0.31)	24.2 (±0.02)	21.0 (±2.33)
M	25.83 (±0.87)	21.43** (±0.29)	26.14 (±1.14)	20.71** (±0.25)	23.0* (±1.59)	27.0* (±0.75)	32.29* (±1.24)	24.86* (±0.88)
O	22.22 (±0.40)	20.50* (±0.50)	21.42 (±0.45)	21.50 (±0.83)	26.6 (±1.03)	28.5 (±0.61)	27.30 (±0.47)	26.0 (±1.04)

Legend: Data presented are the means (± SEM) in grams *p < 0.05, and **p < 0.01, n = 11-17/group).

3.2 Distances travelled

To investigate the distances travelled by Y, M, and O mice over the experimental period we performed linear mixed-effects modelling of the mean daily distances travelled by Y, M, and O mice over the first four weeks (Figure 1A), and the mean daily distances travelled by M and O mice from month one to six (Figure 1B). Analyses of mean daily distances travelled over the first four weeks by Y, M, and O mice found a non-significant interaction between group and time (p = 0.213), suggesting there were no significant differences over the four weeks in the associations between distances travelled and group, and this was confirmed by post hoc analyses with Bonferroni correction for multiple comparisons. Similarly, linear mixed-effects model analyses of mean daily distances travelled in M and O mice over months one to six found no significant group by time interaction (p = 0.867). However, following adjustment for multiple post-hoc comparisons with Bonferroni correction, a significant comparison was found showing M mice demonstrated daily mean distances travelled of 1.189km less than O mice in month four (p = 0.006) (Figure 1B). This difference was largely driven by a significant increase in the distances travelled by M female mice compared to male mice in month 6 (p = 0.012) (Table 2), and was no longer evident in months five or six. Interestingly however, there was an overall non-significant pattern of M female mice running longer distances in the M and O groups. Female M mice travelled greater distances than male M mice in months one, two, three, and five, and female O mice ran greater distances than male O mice in all months except month three, with a pattern of a trend towards significant differences in months seven, eight, nine, and 10 (p = 0.085, p = 0.085, p = 0.067, and p = 0.062) (Table 2). In addition, there were modest reductions in the distances travelled was evident over time in O mice from month four that continued through months seven, eight, nine, and ten, however these were not significant (compared to month four showing the greatest exercise: month 5 p = 0.810; month 6 p >0.786; month 7 p = 0.401; month 8 p = 0.302; month 9 p = 0.244, month 10 p = 0.096, and month 11 p = 0.737), and are therefore unlikely to have effected behaviours.

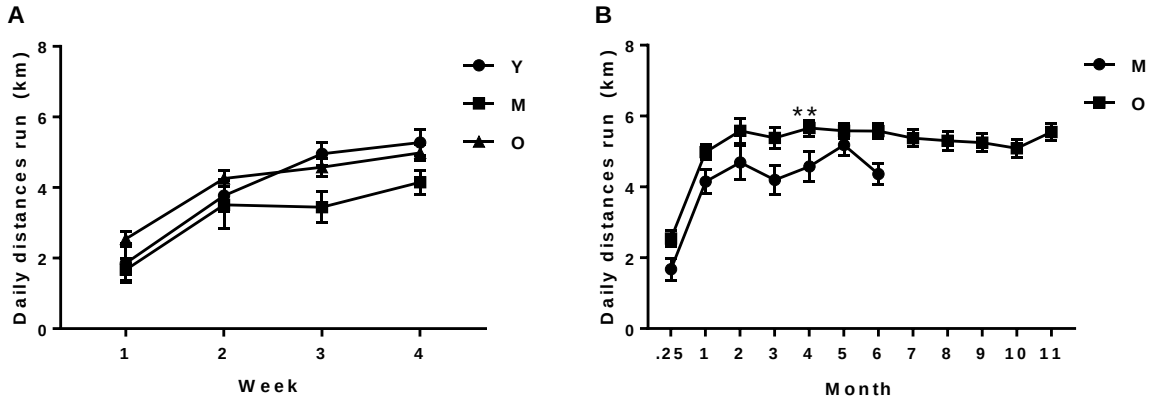

Figure 1. Distances travelled by Y, M, and O C57BL/6 mice over the experimental period. A) Mean daily distances travelled over the first four weeks by Y, M, and O mice, and B) Mean daily distances

travelled over months by M and O mice (Bonferroni post hoc correction for multiple comparisons significance level of 0.007; **p = 0.006 for M vs. O mice, N = 11-17/group).

Table 3. Mean daily distances travelled by Y, M, and O C57BL/6 mice over the experimental period.

Age group	Month										
	1	2	3	4	5	6	7	8	9	10	11
Y	4.421 ± 0.680										
Male	5.927 ± 1.087										
Female	4.953 ± 0.144										
P value	0.231										
M	4.152 ± 0.307	4.689 ± 0.337	4.193 ± 0.416	4.576 ± 0.417	5.181 ± 0.287	4.362 ± 0.307					
Male	4.051 ± 0.168	4.480 ± 0.682	3.948 ± 0.424	4.663 ± 0.395	4.966 ± 0.465	3.597 ± 1.200					
Female	4.253 ± 0.676	4.897 ± 0.705	4.439 ± 0.738	4.489 ± 0.767	5.468 ± 0.259	5.0 ± 0.408					
P value	0.776	0.677	0.573	0.843	0.409	0.0125*					
O	4.986 ± 0.224	5.582 ± 0.360	5.383 ± 0.299	5.661 ± 0.224	5.585 ± 0.218	5.576 ± 0.216	5.378 ± 0.246	5.300 ± 0.262	5.252 ± 0.263	5.091 ± 0.249	5.548 ± 0.248
Male	4.950 ± 0.295	5.316 ± 0.625	5.396 ± 0.396	5.361 ± 0.297	5.284 ± 0.302	5.251 ± 0.303	4.922 ± 0.343	4.888 ± 0.364	4.815 ± 0.370	4.670 ± 0.355	5.275 ± 0.417
Female	5.028 ± 0.359	5.90 ± 0.265	5.386 ± 0.479	6.021 ± 0.320	5.946 ± 0.289	5.966 ± 0.271	5.842. ± 0.343	5.795 ± 0.330	5.777 ± 0.314	5.596 ± 0.285	5.767 ± 0.299
P value	0.867	0.432	0.992	0.146	0.134	0.100	0.085	0.085	0.067	0.062	0.340

Legend: Data presented are the mean +/- SEM (*$p < 0.05$, N = 11-17/group).

3.3 Effects of exercise on behaviours

3.3.1 Baseline locomotion

Baseline locomotion was quantified in the non-stressful environment of the animals' home cages with running wheels removed. Unpaired t-tests found that compared to control mice, exercised mice travelled less distance in the home cage in Y and O mice but not at in M mice ($p = 0.001$, $p = 0.114$, and $p = 0.033$ respectively) (Figure 2A-C). Differences between males and females in the distances travelled were evident in all age groups. Exercise had a gender effect in M and O mice ($p = 0.007$, $p = 0.014$), but not in Y mice (0.180). Post hoc analyses showed M female control mice and O male control mice travelled significantly greater distances compared to their male and female counterparts respectively ($p = 0.001$ and $p = 0.004$).

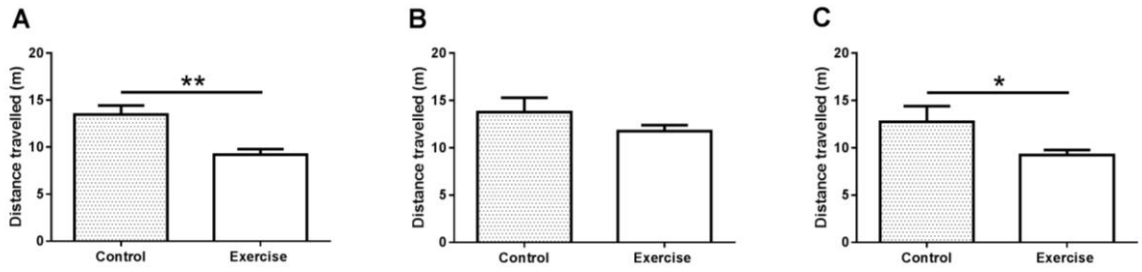

Figure 2. Baseline locomotion for C57BL/6 mice over the lifespan. Distances travelled in the home cage for mice in A) Y mice, B) M mice, and C) O mice (**$p < 0.01$, *$p < 0.05$, N = 11-17mice/group).

3.3.2 Open field

Baseline locomotion was also quantified in the more stressful environment of the Open field. There was no difference in the distances travelled in this environment between control and exercise mice at 4 months ($p = 0.584$) as was found by unpaired t-test, but exercised mice travelled significantly less than control mice in M mice and O mice ($p = 0.0005$, and $p = 0.053$) (Figure 3A-C). There was no effect of sex in distances travelled at any age.

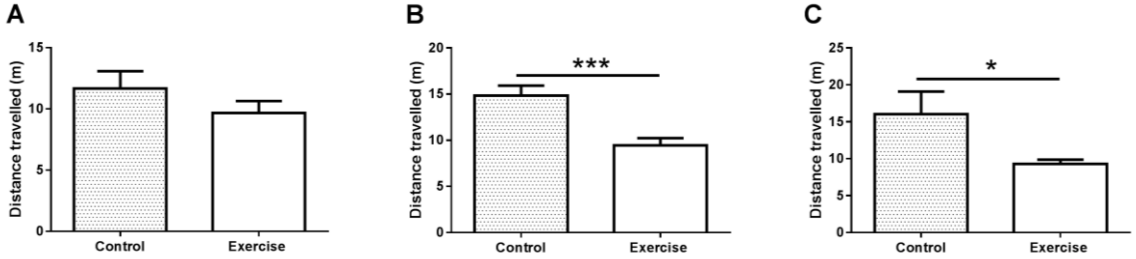

Figure 3. Distances travelled in the Open Field by C57BL/6 mice over the lifespan in A) Y mice, B) M mice, and C) O mice (***$p < 0.001$, *$p = 0.05$, N = 11-17mice/group).

3.4 Anxiety-like behaviours

3.4.1 Open field

The centre regions of the open field are considered anxiogenic, so anxiety-like behaviour was measured by quantifying time spent in the centre of the Open Field. The unpaired t-test found no differences between control and exercise mice in centre time in Y mice ($p = 0.205$) (Figure 4). Compared to control mice, exercised mice spent significantly more time in the centre of the open field in M mice and O mice ($p = 0.021$ and $p = 0.026$). There was no main effect of the animals' sex on time spent in the centre of the open field in Y mice ($p = 0.140$), whereas a significant sex by treatment interaction effect was evident in M mice, and a significant main effect of sex was evident in O mice ($p = 0.025$ and $p = 0.039$). This remained significant in post hoc testing only in M mice, where male exercise mice displayed greater centre time than female exercise mice ($p = 0.027$).

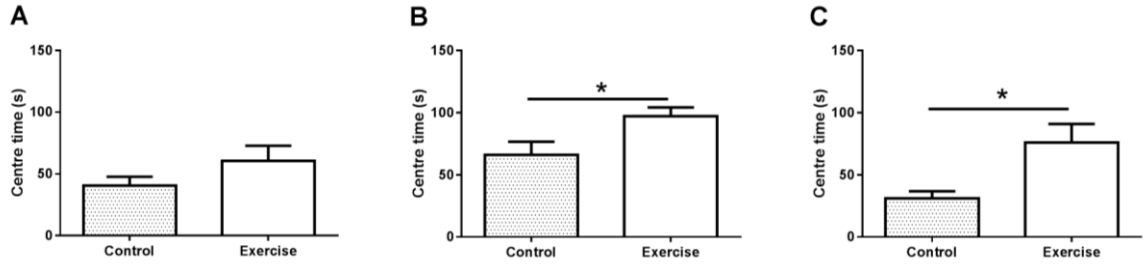

Figure 4. Time spent in the centre of the Open Field for C57BL/6 mice over the lifespan in A) Y mice, B) M mice, and C) O mice (*$p < 0.05$, N = 11-17mice/group).

3.4.2 Elevated zero maze

Anxiety-like behaviours were also assessed by measuring the time spent in the open quadrants of the Elevated Zero Maze. Interestingly, unpaired t-tests showed there were no significant differences between exercised mice and control mice in open quadrant time in Y or O mice ($p = 0.322$ and $p = 0.801$), whereas exercised mice demonstrated significantly greater time in the open quadrants of the EZM in M mice ($p = 0.036$) (Figure 5A-C).

Anxiety-like behaviours were further examined by quantifying head dipping behaviours over the edge of the maze. There were no differences between control and exercise mice in Y or O mice, but exercised mice demonstrated greater head dipping behaviour than control mice in M mice ($p < 0.0001$) (Figure 5D-F). There were no differences between male and female mice for time spent in the open quadrant at any age, or in the number of head dips for Y or O mice. However a significant effect of sex was evident in M exercise mice ($p = 0.005$), and Sidak's post hoc testing found significantly greater head dipping in female exercise mice compared to male exercise mice ($p = 0.021$).

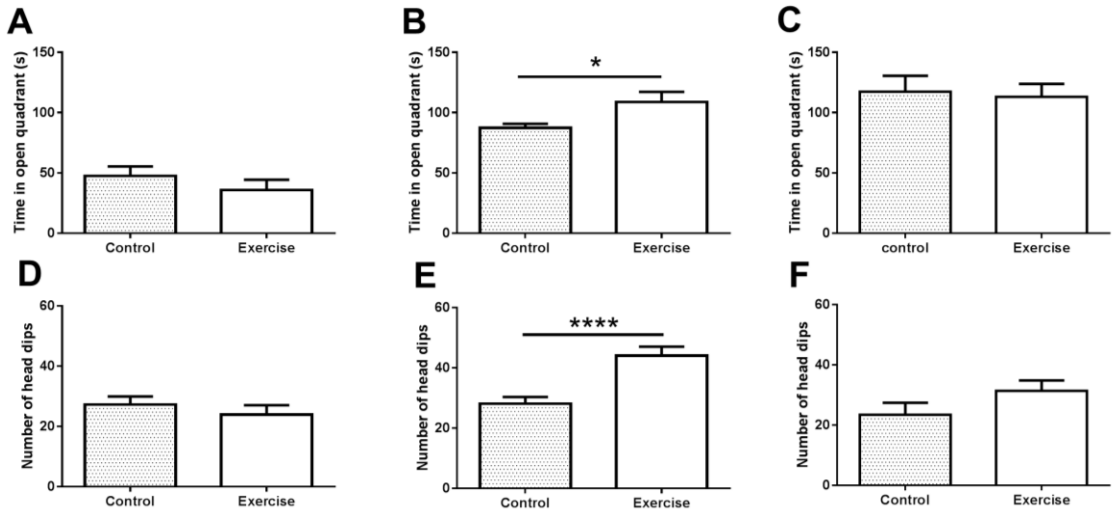

Figure 5. Anxiety-like behaviours in middle aged C57Bl/6 mice. Time in the open quadrants of the Elevated Zero Maze in A) Y mice, B) M mice, and C) O mice. Head dipping over the edge of the Elevated Zero Maze in D), Y mice E), M mice, and F) O mice (* = < 0.05, and ****p < 0.0001, N = 11-17mice/group).

3.5 Depression-like behaviour

3.5.1 Forced swim test

Immobility time in the Forced Swim Test is regarded as a measure of anhedonia or despair, and is thought to be a measure of depression-like behaviour. Contrary to expectations, compared to control mice, exercised mice demonstrated no differences in immobility time at any age (Figure 6). There were also no differences in immobility time found between male and female control or exercise mice at any age.

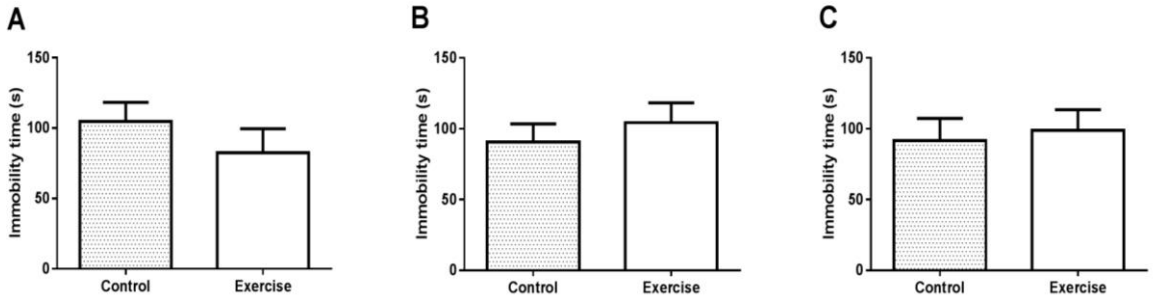

Figure 6. Depression-like behaviour assessed by quantification of immobility time in C57BL/6 mice in the Forced Swim Test in A) Y mice, B) M mice, and C) O mice (N = 11-17mice/group).

3.6 Cognition-like behaviour

3.6.1 Y maze

We investigated the effects of exercise on spatial recognition memory over the lifespan utilising the Y maze. Unpaired t-tests found no differences between control and exercised mice in spatial

recognition memory in Y mice (p = 0.230), however exercise mice displayed an increased preference index in M mice (p = 0.015), that reduced with aging in O mice (p = 0.007) (Figure 7A-C). There was no main effect of sex noted at any age.

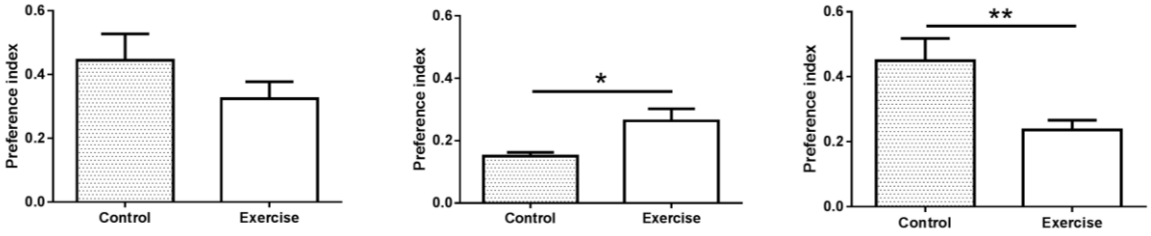

Figure 7. Preference index of recognition memory over the lifespan in A), Y mice B), M mice, and C) O mice (*p < 0.05, **p < 0.01, N = 11-17mice/group).

3.6.2 Barnes maze

The Barnes maze investigated spatial learning, memory, and cognitive flexibility over the lifespan. Mice were assessed on their capacity to learn the location of an escape box over four days of training and the latency to locate the box was taken as a measure of spatial learning. Curiously, Y exercise mice showed longer latencies to the escape box compared to control mice on training days 1, 3 and 4 (p = 0.017, p = 0.011 and p < 0.001) (Figure 8A) however this was largely explained by increased latencies for female mice to locate the escape box on days 2, 3, and 4 (p = 0.026, p = 0.006, and p = 0.0003). There were no differences in training day latencies evident in M mice, but exercise mice displayed increased latencies on day 2 in O mice (p < 0.001)(Figure 8B-C).

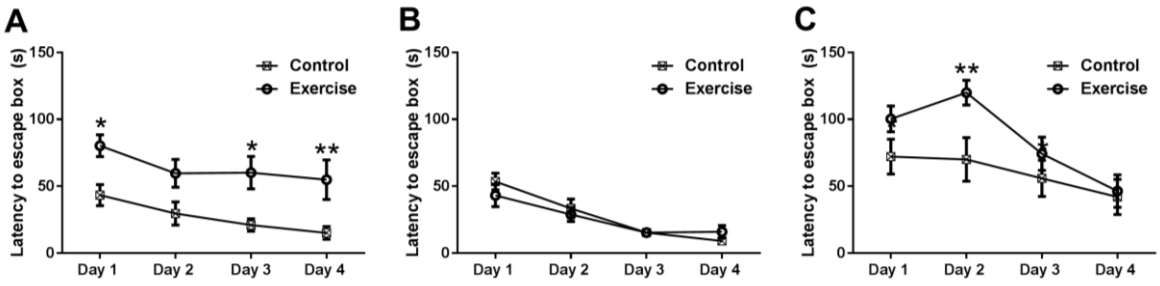

Figure 8. The Barnes maze over four days of training for C57BL/6 mice in A) Y mice, B) M mice, and C) O mice (* = p < 0.05, ** p < 0.01, N = 11-17mice/group).

Following 4 days of training, a probe trial was conducted on day 5 where the position of the training day escape box was rotated clockwise 90 degrees. Latencies to locate the training day escape box location and the new escape box locations were recorded to quantify spatial retention memory and cognitive flexibility respectively. Compared to control mice, exercise mice Y and O mice displayed increased latencies to locate both the original box location and the new box location (Table 4). There were no differences between the sexes at any age to identify the old or the new locations of the escape box in the probe trial.

	Time to find training escape box location (s)		Time to find new escape box location (s)	
	Control	Exercise	Control	Exercise
Y mice	27.627 ± 17.021	68.864 ± 24.255†	69.155 ± 15.837	80.918 ± 20.950
M mice	7.736 ± 1.778	10.454 ± 2.740	62.369 ± 15.987	54.621 ± 8.929
O mice	62.557 ± 21.034	90.441 ± 19.390‡	87.964 ± 18.383	166.610 ± 6.396‡‡

Table 4. Barnes maze probe trial latencies to escape box locations during the Probe trial. ($^{\dagger}p < 0.05$, $^{\ddagger}p < 0.01$, $^{\ddagger\ddagger}p < 0.001$ compared to control mice, N = 11 -17 mice/group).

4. Discussion

This study has investigated the effects of lifetime exercise on anxiety-like, depression-like, and cognition-like behaviours in Y, M, and O mice. Interestingly, although our results revealed exercise reduced OF anxiety-like behaviours in line with our expectations, only M exercise mice displayed reduced anxiety-like behaviours in the EZM, and no exercise related changes to depression-like behaviours were evident. Similarly, whilst our results revealed exercise enhanced recognition memory in M mice, apparently reduced recognition memory was evident in Y and O exercise mice. Although an apparent reduction in recognition memory in O exercise mice and potentially impaired spatial memory in Y exercise mice seems counterintuitive, these behaviours are nevertheless a product of lifetime exercise. We consider that given exercise induced hippocampal neurogenesis is associated with increased anxiety-like behaviours in anxiety provoking tests [32, 33], it is possible that exercise related increases in anxiety were contributing factors to the apparently reduced recognition memory and spatial learning performance in our results.

In addition, our results also revealed control mice displayed elevated levels of baseline home cage locomotion across the lifespan, suggesting potentially pathologically elevated baseline activity levels. Lifetime exercise reduced home cage locomotion in Y and O mice, and reduced locomotion in the novel environment of the OF in M and O mice, with Y female mice travelling further in the home cage than male mice, and O male mice travelling further in the home cage than female mice. Other effects of sex on behaviours were evident predominantly in M mice, with male mice displaying greater centre time than female M mice, and female M mice showing greater head dipping exploration of the EZM than male mice.

3.1 Lifetime exercise alters baseline locomotor activity

Baseline locomotion may impact significantly on behavioural results. Our results revealed control mice show elevated levels of home-cage locomotor activity that remained stable across the lifespan. Consistent with previous work conducted with C57BL/6 mice, Y exercise mice showed reduced home cage locomotor activity [10], although this did not reach significance in M mice. However, M exercise mice have exercised from 12 weeks until nine months of age – the six month duration of the adult lifespan, and therefore demonstrate all the health benefits of regular exercise at this age. In particular, this is likely to include exercise induced upregulation of the expression of Sirtuin 1 (Sirt 1) that activates PGC1a (peroxisome-proliferator-activated receptor-γ or PPARGC1A) to orchestrate mitochondrial biogenesis and mitochondrial copy number [34-38]. Upregulated mitochondrial biogenesis allows for greater mitochondrial respiratory chain function that enables ATP production for cellular functioning to meet the body's energy needs. It is possible that this may have contributed to greater home cage locomotor activity in M mice that was non-significantly different from M control mice. Moreover, whilst ageing involves declines in mitochondrial biogenesis and exercise can ameliorate such declines [39, 40], it seems reasonable that increases in home cage

locomotor activity to control levels would not be anticipated in O mice, in agreement with our results.

It is also noteworthy that Y female mice elected to travel significantly greater distances in the home cage than Y male mice. This could suggest that Y female mice require greater baseline levels of physical activity than Y male mice to maintain health during young adulthood, and raises some interesting issues in the context of anxiety-like, depression-like, and cognitiion-like behaviours in the context of ageing. The female sex hormone estradiol (E2) has significant effects on stress, with higher levels of E2 resulting in greater psychosocial stress than lower levels, and may be a mechanism involved in the prevalence of affective disorders in women [41]. Furthermore, E2 interacts with exericse. Exercise induced neurogengesis is transiently increased with estogen [42], and wheel running for 1 or 6 months in middle aged female C57BL/6 mice induces similar levels of hippocampal neurogenesis and improves spatial memory [43]. Exercise also has comparable effects to E2 on attention and memory [44]. Furthermore, exercise may be more effective at attenuating stress hormones if estrogen is present [18]. Given these factors, it may be that female mice self-select levels of exericse that are optimal for balancing and maintaining healthy levels of female sex hormones, hippocampal neurogensis, and adaptive behaviours. Given that depression and cognitive impairment during ageing is more prevalent in human females than males, additional well powered studies are celarly needed to investigate and elucidate these issues.

Long-term exercise in Y mice had no effect on locomotor activity in the novel context of the open field, and this is consistent with previous findings of a lack of correlation between exercise and locomotor activity in the open field (during the light phase) in young adult mice at 8 weeks of age (r +0.09, ns) [45]. However reduced activity was evident with exercise in M and O mice, contrary to results from previous studies showing exercise related increases in activity in this test [46, 47], however species (mouse cf. rat) and running protocol (running wheel cf. treadmill running) are likely to account for these differences. Reduced locomotor activity in the OF is thought to be indicative of anxiety-like behaviour [48], suggesting that the reduced locomotor activity in M and O mice may be related to exercise related increases in anxiety in this test.

3.2 Exercise associated neurogenesis may increase anxiety and impair cognition-like function

There is potentially further evidence of exercise related anxiety in our results. Work by Fuss et.al. [49] has shown that chronic wheel running induced hippocampal neurogenesis in single housed male C57BL/6J mice had large correlations with markers of anxiety-like behaviours such as the total distance travelled in the light-dark box (r -0.78, p = 0.001) and rearings in the open field (r -0.67, p = 0.011). Onksen et.al [32] later further demonstrated that both male and female singly housed mice homozygous for the Cre/lox -conditional allele of ATR ($ATR^{f/f}$) (ataxia telangiectasia-mutated and rad-3-related protein; a cell cycle kinase necessary for normal levels of hippocampal neurogenesis) displayed increases in anxiety-like behaviours of reduced open field centre time and distance travelled in the light compartment of the light-dark box following 4 weeks of wheel running. Although mice in the present study were socially housed, these findings are suggestive of a mechanism that plausibly explains increases in anxiety-like behaviours arising from exercise in the present study.

Interestingly, and contrary to our expectations, there was no exercise related improvement in recognition memory in Y mice – indeed a non-significant reduction was evident. Further investigations revealed no significant differences between Y control mice and Y exercise mice in the distances travelled or the time spent freezing in the maze, so the reasons for this reduction are unclear, and are contrary to previous work finding exercise related enhancement of recognition memory in adult mice aged 12 weeks (mediated by increases in neurogenesis) [50]. Our investigation

into the effects of lifetime exericse in M mice revealed M exercise mice displayed signifcantly better recognition memory than M control mice at this age, in agreement with our expectations. Whilst there are a paucity of studies examining the effects of exericse on recognition memory in M and O mice, it seems likely that increases in hippocampal neurogenesis would be involved in mediating exercise effects. It is possible that exercise associated in increases in hippocampal neurogenesis contributed to a significant reduction in recognition memory in O exercise mice comared to control O mice. However it is possible that exercise related fatigue also contributed to the reduction in exploration of the novel arm of the Y maze in this age group, or that the combination of these factors contributed to impaired performance. Examination of the distances travelled by O mice in the Y maze revealed O exercise mice travelled significantly less distance than control O mice ($p = 0.014$). Given that reductions of locomotor activity in novel contexts is considered to reflect increased anxiety-like behaivour [48], significantly reduced distance in the Y maze by O exercise mice is suggestive of an exercise related increase in neurogenesis that may have contributed to greater anxiety, with reduced exploration of the maze that subsequently limited cognitive performance in this test. This may also have been effected by exercise related fatigue. Additional work is clearly required to elucidate whether exercise related neurogenesis is correlated with rodent performances in other tests where anxiety might confound results, such as the EZM and Barnes maze indices of spatial learning, memory, and cognitive flexibility.

Our investigations into the effects of lifetime exericse on spatial learning in the Barnes maze revealed behaviours that were contrary to our expectations. We revealed poorer performance in spatial learning and spatial memory in Y exercise mice compared to Y control mice on days one, three, and four, and no effect of exercise in M or O mice with the exception of longer latencies to locate the escape box for O exericse mice on day two of training. Exercise has been demonstrated to increase hippocampal neurogenesis in both adult and ageing mammals [12, 21], so it seems likley that our unanticipated results are related to the effects of lifetime exericse. Whilst there were no differences in the distances to the escape box in Y exericse mice compared to Y control mice, Y exercise mice displayed longer freezing times in all four training days (day 1 33.356 ± 7.581 vs 57.663 ± 10.071; day 2 18.571 ± 4.182 vs 34.003 ± 7.754 ; day 3 23.681 ± 4.768 vs 42.551 ± 10.253; and day 4 25.141 ± 5.457 vs 30.262 5.290, $p = 0.002$) suggesting exercise related neurogenesis contributed to greater anxiety-like behavours that increased the latencies to the escape box and may have impaired spatial learning in this test. Interestingly, female latencies to the escape box were significantly longer than males on days two, three, and four, suggesting that overall, exercise may have contributed to greater anxiety that may have extended the escape box latencies for Y female mice, thereby increasing the mean latencies for the Y exericse group. This hypothesis was confirmed by two way ANOVA of sex differences in freezing time that showed significant effect of sex ($p < 0.003$) with post hoc analyses revealing significantly greater freezing by Y female mice compared to Y male mice on all four training days (all $p < 0.0001$). Whilst exercise related increased anxiety may have impaired spatial learning in Y exercise mice, exercise reduced anxiety in M mice, and this appears to have resulted in reductions in latencies to the escape box that were comparable to M control mice levels, although exercise did not enhance spatial learning beyond that shown by M control mice.

The probe trial investigations into spatial memory and cognitive flexibliity revealed that Y exercise mice displayed longer latencies to identify the locations of the old and new boxes as measures of spatial memory and cognitive flexibility respectively, suggesting that anxiety may have also contributed to impairment in these meaures. Interestingly, analysis of the distances travelled between control and exercise mice in the Probe trial revealed that Y exercise mice travelled significantly less than Y control mice consistent with reductions in home cage locomotor acitivty, and suggesting that neurogenesis associated anxiety is likely to have impacted on spatial memory performance in Y exercise mice. O exercise mice demonstrated singificantly longer latencies to identifiy the location of the new escape box as a measure of cognitive flexibility. Further t-test investigations into the distances travelled and freezing time in this test revealed no significant differences in the distances travelled ($p = 0.090$) but a significant increase in freezing time for O

exericse mice compared to O control mice ($p = 0.001$), suggesting exercise related anxiety associated with hippocampal neurogenesis may have persisted into old age and affected cognitive flexibility in O mice.

3.3 Chronic exercise can reduce anxiety-like, depression-like, and cognition-like impairment behaviours

Our results of no lifetime exercise related changes in depression-like behaviours and potential impairments to cognition-like behaviours belies the notable range of advantageous behavioural, cellular, and molecular adaptations associated with exericse suggesting that lifetime exericse may be beneficical and attenuate ageing related anxiety, depression, and cognitive impairment. Chronic exercise has long been considered to reduce emotionality [51], however recent studies overwhelmingly show chronic exercise can reduce anxiety-like [52] and depression-like behaviours [10, 14] with even low intensity exercise [53]. Exercise can also normalise and improve cogntion-like function and impairment [43], and has protective effects against cognitive decline in ageing mice [54]. However the increasing interest in the therapeutic effects of exericse has revealed inconsistencies, with occasional studies finding exercise induced increases in anxiety that seem to be associated with neurogenesis levels as was noted above [49, 55, 56]. Nevertheless, chronic exericse also confers resilience to stress induced anxiety-like and depression-like behaviours arising from trauma and post traumatic stress [57, 58], and cognitive-like changes arising from early life stress [59]. Lifestyle related stressors such as sleep deprivation related memory impairment [60], smoking cessation associated stress [61], and the detrimental effects of a high fat diet on memory during ageing [62] are also reduced with chronic exericse.

The peripheral benefits of chronic exercise include the maintenance of lean body mass during ageing (the prevention of sarcopenia) [63], and reductions in resting glucose, triglycerides, and cholesterol levels [64]. There are also central nervous system (CNS) benefits such as increases in cortical capillary volume, capillary length, and volume [65], greater blood flow in limbic brain regions following chronic stress pathophysiology [66], and the attenuation of HPA axis responses to psychological stressors [67]. Chronic exercise involves brain region specific changes in physiology that modulates cardiovascular, circadian, and metabolic status [68], and may be protective of telomere length that is considered a marker of cellular ageing [69, 70]. There is also well known exercise related upregulation of neurotrophins and signalling proteins including NGF (nerve growth factor), BDNF (brain derived neurotrophic factor), and TrkB (*Tropomyosin receptor kinase B*) that are required for the survival of new neurons in the hippocampus [71-73] and are associated with enhanced spatial learning and recognition memory [74, 75]. Interestingly, whilst a blocked wheel increased cell proliferation, only running increased the number of new neurons in a dose dependent manner [76], with increases in new neuron numbers of 200% in exercising mice [77] that may be a contributing factor to reducing CA1 and dentate gyrus atrophy following stress [78]. Finally, exercise is increasingly demonstrating inter-generational benefits such as enhancing learning and memory in the offspring of both maternal and paternal exericsing parents [79, 80]. In contrast to this range of benefits from exercise, the available evidence about the effects of cessation of exericse in the brain from mouse studies shows the cessation of chronic exercise increases anxiety-like behaviours and cognitive-like impairment with reductions in BDNF and hippocampal neurogenesis [81, 82], suggesting that exercise may need to be sustained to maintain its benefits for the CNS during ageing.

3.4 Conclusions

In conclusion, to the authors' knowledge, this study is the first to investigate the impacts of lifetime exericse in normal healthy aging mice over the lifespan. Our results support the hypothesis that lifetime exericse reduces (OF) anxiety-like behaviours in M and O mice. Interestingly, and contrary to our expectations, we reveal no exercise related changes in deprssion-like behaviours, and possible

exercise related impairment to spatial learning in Y mice, that largely normalised in M and O mice, however no exercise induced improvements were evident compared to control M and O mice. However interestingly, our results also suggest exercise related increases in anxiety-like behaviours that are likely due to exercise associated increases in hippocampal neurogenesis, consistent with the findings of previous studies [32, 33]. We reveal likely exercise related anxiety-like behaivors of reduced distances travelled in O mice and freezing in Y mice that appear to have contributed to the unanticipated reduced recognition memory and spatial learnning respectively. These findings highlight that the effects of lifetime exercise are not simply an extension of the effects of chronic exercise. Additional well powered studies are required to elucidate the impacts of lifetime exercise on anxiety-like, depression-like, and cognition-like behaivours, the associated mechanisms involved, and the sex specific effects of exercise for female mice in particular given the prevalence of depression and dementia in ageing human females.

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6. Declaration of interest

The authors have no actual or potential conflicts of interest to disclose that could inappropriately influence, or be perceived to influence this work.

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