

Initial assessment of the spatial learning, reversal, and sequencing task capabilities of knock-in rats with humanizing mutations in the A β -coding region of *App*

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Abstract

Model organisms mimicking the pathogenesis of human diseases are useful for identifying pathogenic mechanisms and testing therapeutic efficacy of compounds targeting them. Models of Alzheimer's disease and related dementias aim to reproduce the brain pathology associated with these neurodegenerative disorders. Transgenic models, which involve random insertion of disease-causing genes under the control of artificial promoters, are efficient means of doing so. There are confounding factors associated with transgenic approaches, however, including target gene overexpression, dysregulation of endogenous gene expression at transgenes' integration sites, and limitations in mimicking loss-of-function mechanisms. Furthermore, the choice of species is important, and there are anatomical, physiological, and cognitive reasons for favoring the rat over the mouse, which has been the standard for models of neurodegeneration and dementia. We report an initial assessment of the spatial learning, reversal, and sequencing task capabilities of knock-in Long-Evans rats with humanizing mutations in the A β -coding region of *App*, which encodes amyloid precursor protein (*App*^{h/h} rats), using the IntelliCage, an automated operant social home cage system, at 6-8 weeks of age, then again at 4-5 months of age. These rats were previously generated as control organisms for studies on neurodegeneration involving other knock-in rat models from our lab. *App*^{h/h} rats of either sex can acquire place learning and reversal tasks. They can also acquire a diagonal sequencing task by 6-8 weeks of age, but not a more advanced serial reversal task involving alternating diagonals, even by 4-5 months of age.

Introduction

Technical innovation has enabled researchers in the past two decades to study neurodegenerative disorders with greater precision. For example, optogenetics, a technique for modulating individual neuron activity through activation of light-sensitive proteins called opsins(1), has been used to evaluate grafts of mesencephalic dopaminergic neurons derived from human embryonic stem cells in a mouse model of Parkinson's disease(2); while electron cryo-microscopy, whose resolution has become comparable to that of X-ray crystallography(3) has revealed the structure of

histopathological tau filaments in patients with frontotemporal dementia(4). Single-cell resolution transcriptomics has been used to characterize the cell diversity of the entire mouse central nervous system (CNS)(5), yielding reference data for exploring the molecular mechanisms of Alzheimer's disease (AD), the most common form of dementia among the elderly, and related dementias in the context of animal models, which have diversified following improvements in genome-editing technologies(6). In parallel with model diversification arose high-throughput, automated methods for behavioral phenotyping(7): alongside traditional paradigms such as novel object recognition, the Morris swim task (also known as the Morris water maze), the T-maze, and the Y-maze, are operant touchscreens(8) and systems like the IntelliCage (NewBehavior AG)(9, 10), which has been used to identify cognitive deficits in multiple mouse models of AD in a socially housed setting(11).

Despite these advances and decades of research overall, the pathogenic mechanisms of AD remain poorly understood and no viable treatments have been developed(12), suggesting a fundamental flaw in the investigative approach, the underlying pathogenic assumption, or both. In this case, there is evidence implicating both: most AD research has focused on the role of amyloid- β peptide (A β) accumulation, either as insoluble aggregates or soluble dimers/oligomers as the causative agent of a series of events involving neuronal death, oxidative stress, synaptic loss, and neuroinflammation leading to AD, which defines the "amyloid cascade hypothesis"(13, 14). This, encouraged by links between autosomal dominant mutations in genes causing AD and changes in proteolytic processing of APP, or amyloid precursor protein(15), has prompted researchers to generate animal models of overexpression, mostly transgenic mice, as an efficient way of reproducing these alleged pathogenic conditions(16). However, individuals can have significant amyloid plaque burden without corresponding memory impairment(17-22), and many models do not exhibit the widespread neurodegeneration, cortical atrophy, or neurofibrillary tau tangles present in AD(13, 16). Moreover, the transgenic approach is non-physiological and etiologically biased, which is not ideal for simulating the human disease state.

The species also matters in evaluating the animal model paradigm. Compared to mice, rats have larger brains, which allows for more accurate direction of cannulas (for administration of drugs,

biologics, viruses, etc.) and micro-dialysis probes (for sampling extracellular brain levels of neurotransmitters, A β , soluble tau, etc.) to individual brain regions, causing less damage and increasing specificity. In vivo brain imaging techniques, such as MRI(23) and PET(24-26), can assess the extent and course of neurodegeneration with better spatial resolution in rats. Moreover, rats are large enough for convenient in vivo electrophysiological recordings or serial sampling of cerebrospinal fluid for detection of biomarkers. Rats also express in the brain during adulthood due to alternative splicing, like humans(27), both three- (3R) and four-repeat (4R) microtubule-binding domain isoforms of tau(28-31), a protein that forms neurofibrillary tangles in AD and is mutated in frontotemporal dementia(32-39), unlike adult mice, which only express 4R isoforms(40). Finally, rats have been a choice species for behavioral, memory, and cognitive research due to their physiological similarity with humans and intelligence(41-44), which are critical when studying neurodegenerative diseases. These observations, along with the failure to translate results from current models into viable therapies(12), suggest that knock-in rat models may be better suited for the study of mechanisms underlying neurodegeneration and dementia than transgenic mouse models.

We report an assessment of the spatial learning, reversal, and sequencing task performance of knock-in Long-Evans rats carrying humanizing mutations in the A β -coding region of *App* (*App*^{h/h} rats) at 6-8 weeks of age, and again at 4-5 months of age, with the IntelliCage. As human and rat A β differ by three amino acids and A β aggregates are widely regarded as the main pathogenic molecules in AD with human A β being possibly more likely to form toxic A β species than rodent A β , (45) these animals are controls for knock-in rat models designed and generated by L. D'Adamio for neurodegeneration research, which have already yielded insights on biochemical effects of pathogenic(46) and protective(47) mutations in *APP*(48, 49), the pathogenic *PSEN1* L435F mutation(50), the Familial Danish ITM2b mutation(51), and of the R47H variant in *TREM2*(52-54), which is associated with increased AD risk (55, 56). Full characterization of these models requires cognitive and behavioral evaluation: this study establishes in part a baseline profile for the control rats while exploring analytic ideas specific to the IntelliCage paradigm that may inform further work, specifically activity curves representing aggregate behavior of experimental subjects and use of the

area under those curves as a measure for statistical comparison. Moreover, the body of IntelliCage research is small: a PubMed query in December 2021 for “IntelliCage” yielded only 128 results dating from 2005, with the majority referring to mouse experiments. Therefore, this study is not only an initial cognitive characterization, but also a contribution to the nascent body of rat IntelliCage literature and a reference for analytical methods.

Material and Methods

Animals and Experimental Design

All experiments were done according to policies on the care and use of laboratory animals of the Ethical Guidelines for Treatment of Laboratory Animals of the NIH. Relevant protocols were approved by the Rutgers Institutional Animal Care and Use Committee (IACUC) (Protocol #201702513). All efforts were made to minimize animal suffering and reduce the number of rats used. Prior to and after behavioral analysis, males were housed 2 per cage and females were housed 3 per cage under controlled laboratory conditions with a 12-hr dark/light cycle, at a temperature of $25 \pm 1^\circ\text{C}$. They were anesthetized with isoflurane, tagged subcutaneously with radio frequency identification transponders, and allowed to recover for at least a week. Rats had free access to standard rodent diet and tap water while in traditional housing and were monitored for dehydration during periods of water restriction during behavioral analysis. Long-Evans rats expressing humanized *App* alleles (*App*^{h/h}) were generated as described previously.(48)

IntelliCage

115 The IntelliCage for Rats (NewBehavior AG) was used to collect behavioral data. It consists of a
 116 central square home cage connected to four operant learning chambers, or *corners*. Every corner has
 117 two sides, each with a drinking bottle gated by a rotating door with a nosepoke sensor (Figure 1). The
 118 sides also include LEDs and air puff delivery valves as additional conditioning components.
 119 Behavioral programs are defined by the user within a visual coding platform. Subcutaneously injected
 120 transponders allow the IntelliCage to track the behavior of individual animals with unique radio
 121 frequency identification tags. Among the parameters tabulated for subsequent analysis are corner

Day	Cohort A	Cohort B
1	Free adaptation	Free adaptation
2	Nosepoke adaptation	Nosepoke adaptation
3	Time adaptation	Time adaptation
4		Single corner restriction
5		
6		
7	Place learning	Place learning with corner switch
8		
9		
10	Place reversal	
11	Behavioral sequencing	Behavioral sequencing
12		
13		
14		
15		
16	Serial reversal	Serial reversal
17		
18		
19		

Table 1. IntelliCage program timeline overview for cohorts A and B.

122 visits, visit lengths, visit times, number of nosepokes per visit, and number of bottle licks per visit. This
 123 system offers a variety of advantages over human observation: high-throughput, unbiased data
 124 collection and reporting; minimal risk of human error; minimal perturbation of testing conditions; and
 125 uniform testing of multiple animals simultaneously in a social setting. Two cohorts of *App^{h/h}* rats were
 126 studied, A and B, housed across four IntelliCages, separated by sex and cohort. They were run on
 127 separate program timelines, once at 6-8 weeks of age and again at 4-5 months of age (the *first pass*

128 and *second pass* through the program timeline, respectively), as outlined in Table 1 with program
129 descriptions in Figure 2.

130 **Figure 1. IntelliCage schematic.** Central home cage and four labeled corners with two drinking sides per
131 corner.

132 **Figure 2. Descriptions of IntelliCage programs.** (a) Single corner restriction (Cohort B only, 2 days).
133 Progression of drinking (green) and non-drinking (yellow) corner layouts over two days. (b) Place learning
134 (Cohort A only, 3 days). Example of correct (green) and incorrect (yellow) corner layout for a rat assigned to
135 corner 4. (c) Place learning with corner switch (Cohort B only, 4 days). On the top left is the cycle of correct
136 corners with movement every 45 minutes. The rest of the panel, starting from the bottom left, depicts an
137 example of correct (green) and incorrect (yellow, or red highlighting the previously correct corner) layouts for a
138 rat initially assigned to corner 1 and their cycle over the four phases of a drinking session, which ends with the
139 top right layout before returning to the top center layout during the start of the next drinking session. (d) Place
140 reversal (Cohort A only, 3 days). Example of correct (green) and incorrect (yellow, or red highlighting the
141 previously correct corner) corner layout for a rat assigned to corner 4 during place learning. (e) Behavioral
142 sequencing (Cohort A: 3 days, Cohort B: 5 days). Example of correct (green), lateral (yellow), and opposite (red)
143 corner layouts and pattern for a rat initially assigned to either corner 2 or 4. (f) Serial reversal (both cohorts, 4
144 days). Schematic of correct (green), lateral (yellow), and opposite (red) corner layouts and pattern. The starting
145 layout depends on the initial corner assignment.

146 *IntelliCage programs*

147 Free adaptation (both cohorts, 1 day) - The rats may drink water ad libitum and explore the
148 IntelliCage, familiarizing themselves with its layout; all bottle access doors open in response to any
149 corner visit.

150 Nosepoke adaptation (both cohorts, 1 day) - The rats learn they must activate a nosepoke sensor to
151 open a water access door at any corner for seven seconds; this nosepoke mechanic remains active
152 for every program hereafter.

153 Time adaptation (Cohort A: 4 days, Cohort B: 2 days) - The rats may only drink between 8pm and
154 11pm at any corner, a time window called the *drinking session*.

155 Single corner restriction (Cohort B only, 2 days) - All rats must drink from a single correct corner with
 156 the other corners being neutral during the drinking session. The correct corner changes after ninety
 157 minutes, such that the rats can drink at one corner during the first half of the drinking session and
 158 must switch to the opposite corner during the second half. Over two days, the correct corner
 159 designation follows the path 1->3 (1st day), then 2->4 (2nd day), covering all corners (Figure 2A).
 160 Place learning (Cohort A only, 3 days) - The rats may only drink during the drinking session at a
 161 corner assigned to each of them; these assigned corners are considered correct, and the non-
 162 assigned corners are considered incorrect (Figure 2B).
 163 Place learning with corner switch (Cohort B only, 4 days) - Each rat is assigned an initial correct
 164 corner where it can drink during the drinking session, as in place learning, with the other corners
 165 being incorrect. Every 45 minutes, the correct corner designations are switched according to the cycle
 166 (1->3->4->2[->1]). [Figure] illustrates this for a rat with corner 1 as the initially assigned correct corner.
 167 If corner 2 were the initial correct corner, the cycle would be shifted over once (2->1->3->4[->2]). After
 168 the first switch, the positions of the incorrect corners adjust accordingly. By the first 45-minute block of
 169 the next drinking session, the correct corner will have returned to its initial location. A *phase* refers to
 170 a 45-minute block during the drinking session in this program. The end of a phase marks when a
 171 corner switch occurs (Figure 2C).
 172 Place reversal (Cohort A only, 3 days) - The rats may only drink during the drinking session at the
 173 corner diagonally opposite the one assigned in place learning; those reversed corners are considered
 174 correct, and the remaining corners, including the original assigned corner, are considered incorrect
 175 (Figure 2D).
 176 Behavioral sequencing (Cohort A: 3 days, Cohort B: 5 days) - The rats must alternate between
 177 drinking at the initial learned corner and the opposite corner during the drinking session, so that one
 178 corner in the assigned diagonal is active (correct) at a time while the other is inactive (opposite); the
 179 conditions of the corners in the assigned diagonals alternate between correct and opposite, with a
 180 correct nosepoke triggering a condition switch. Visits to corners in the non-assigned diagonal are
 181 considered *lateral* visits (Figure 2E).

182 Serial reversal (both cohorts, 4 days) - The rats must alternate between a behavioral sequencing
183 pattern on the original diagonal and the same on the other diagonal during the drinking session; the
184 diagonal switches after every eight successive correct nose pokes. The corner conditions change as in
185 the behavioral sequencing program, only now one must consider which diagonal is active in
186 determining the corner conditions at any time (Figure 2F).

187 *Corner rank comparison*

188 We followed this workflow to compare animal activity during the single corner restriction program:

189 1. For each 90-minute block of the program during the drinking session, rank the animals according to
190 the number of visits made to the appropriate correct corner; there should be four lists for each
191 IntelliCage, corresponding to the 8:00-9:30pm and 9:30-11:00pm periods of drinking sessions 1 and
192 2.

193 2. An animal is said to *out-visit* another animal at a corner if it makes more visits than the other one
194 during a given time interval. Assign four scores to each animal equal to the number of animals it out-
195 visits, one score for each 90-minute block; each corner should be represented once as a correct
196 corner.

197 3. Use the scores to generate mean scores and standard errors for statistical analysis.

198 *Activity curves*

199 We followed this workflow to produce the activity curves for each program:

200 1. Categorize each rat's visits by their contextual value in the program, i.e., correct, incorrect, lateral,
201 or opposite.

202 2. For each subset of rats by sex, cohort, and pass (e.g., cohort A males in their first pass), count the
203 total number of visits those rats made for each drinking session. For a 3-day program, there should be
204 3 totals for a given subset.

3. For each subset as described in step 3, tabulate the fractional accumulation of visits by category over time for each drinking session, adding to each fraction, starting from 0, the value of 1 divided by the associated total count for that subset and drinking session each time a visit belonging to a certain category occurs, and 0 otherwise. For each drinking session of a given subset, the final fractions should sum to 1.

4. Match each fraction with a timestamp relative to the start of the first drinking session of a program, excluding time not belonging to a drinking session, e.g., if the n th fraction is associated with a visit that occurred during the 35th minute of the third drinking session of a program, the fraction is matched with minute 395 ($180 + 180 + 35$).

5. Plot the resulting tables with time as the independent variable and fraction as the dependent variable, yielding the activity curves.

Statistical analysis

We followed this workflow for statistical analysis of activity for each program:

1. Tabulate the fractional accumulation for individual rats as described above; in other words, make the calculations necessary to generate activity curves for each rat in the IntelliCage rather than a group of them for every drinking session.

2. Calculate the area underneath each activity curve, bounded on the left and right by the start and end of each drinking session, respectively, and below by the x-axis. Before calculating the area, we completed the curve by extending it horizontally such that the final fraction at the end of the drinking session is equal to the fraction accumulated by the last visit the animal made during that drinking session. Each result is a data point representing the cumulative activity of a specific rat for a given drinking session and visit category.

3. Run statistical tests with those data points based on desired comparisons.

Results

***App^{h/h}* rats do not visit corners more often than other *App^{h/h}* rats during single corner restriction in cohort B at either 6-8 weeks or 4-5 months of age.**

After IntelliCage adaptation as outlined in Table 1, rats in cohort B were started on the single corner restriction program, which tested whether the animals were able to share this corner equally among themselves for water. The animals were assigned a rank during each 90-minute block of the two drinking sessions (four ranks total) based on visits to the actively correct corner, as described in the methods. The mean rank was used to compare animal activity (Figure 3). There were no significant differences among male rats during either pass. During the first pass one female rat (Animal 22) had a mean rank significantly lower than that of two other female rats (Animals 16 and 17), but this difference was not observed during the second pass.

Figure 3 – Single corner restriction, cohort B. Average scores for individual animals in cohort B by sex and pass, in decreasing order from left to right. Data points from the first and second passes are indicated by white and red circles, respectively. All data represented as mean \pm SEM ($*p < 0.05$). See Table 2 for statistical analysis. FP = first pass (6-8 weeks), SP = second pass (4-5 months). ♀ = female, ♂ = male.

Ordinary one-way ANOVA, single corner restriction, cohort B			
Female	Pass	F (DFn, DFd)	P
	First (6-8 weeks)	F (9, 30) = 3.306	0.0065
	Second (4-5 months)	F (9, 30) = 0.9019	0.5359
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	16 (1 st pass) vs. 22 (1 st pass)	*	0.0278
	17 (1 st pass) vs. 22 (1 st pass)	*	0.0278
Male	Pass	F (DFn, DFd)	P
	First (6-8 weeks)	F (10, 33) = 0.8408	0.5941
	Second (4-5 months)	F (10, 33) = 0.9633	0.4921

Table 2 – Statistical analysis of data shown in Figure 3 for single corner restriction, cohort B. A p -value less than 0.05 is considered significant ($*p < 0.05$).

***App^{h/h}* rats in cohort A can acquire a place learning task by 6-8 weeks of age with session-wise improvement.**

242 After IntelliCage adaptation as outlined in Table 1, rats in cohort A were started on the place learning
 243 program. Animal activity in this program and subsequent programs was summarized via (1) activity
 244 curves showing the fractional accumulation of corner visits by category of all animals during each
 245 drinking session, organized by sex and pass; and (2) comparisons of mean area under the activity
 246 curves of individual animals by visit category during each drinking session, to quantify differences in
 247 task performance. Qualitatively, an activity curve for correct visits that steepens as the one for
 248 incorrect visits flattens, session-wise, signifies task acquisition (Figure 4A). Analysis of area under the
 249 curves (AUC) revealed significant session-wise increases for correct visits (C-AUC) with
 250 accompanying decreases for incorrect visits (IC-AUC) for both sexes during the first pass (Figure 4B).
 251 During the second pass, there were no significant session-wise differences in C-AUC for either sex,
 252 and IC-AUC was significantly lower for the 3rd drinking session compared to the 1st and 2nd for females
 253 alone (Figure 4B). For the 2nd drinking session of the first pass, IC-AUC was significantly lower for
 254 males (Figure 4B). For females, C-AUC was significantly higher for each drinking session of the
 255 second pass compared to the first, whereas IC-AUC was significantly lower for the 1st drinking session
 256 during the second pass; there were no significant differences between passes for males (Figure 4C).

Figure 4 – Place learning, cohort A. (A) Activity curves showing the fractional accumulation (y-axis) of visits over drinking session time (x-axis), reset every 180 minutes, by sex and pass. Curves for correct and incorrect visits are black and blue, respectively. **(B)** Sex comparison of area under the curve (AUC) for activity curves of individual animals by visit category and pass. “*” denotes significant comparisons within sex across program days, while “#” denotes those for the corresponding program day between sexes. Female (♀) and male (♂) data points are indicated by white and black circles, respectively. **(C)** Pass comparison of AUCs for activity curves of individual animals by sex, program day, and visit category. Data points from the first and second passes are indicated by white and red circles, respectively. All data represented as mean ± SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, similarly for # $p < 0.05$, etc.). See Tables 3-5 for statistical analysis. FP = first pass (6-8 weeks), SP = second pass (4-5 months), C = correct (visits), IC = incorrect (visits).

Figure 4B	Mixed-effects analysis, place learning, cohort A, first pass		
Correct (C-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (2, 43) = 4.389	0.0184
	Day	F (2, 43) = 24.08	<0.0001
	Sex	F (1, 22) = 3.847	0.0626
	post-hoc Sidak's multiple comp. test	Summary	Adjus. P
	Female, day 1 vs. day 2	ns	0.9995
	Female, day 1 vs. day 3	***	0.0010
	Female, day 2 vs. day 3	**	0.0013
	Male, day 1 vs. day 2	***	0.0003
	Male, day 1 vs. day 3	****	<0.0001
Incorrect (IC-AUC)	Male, day 2 vs. day 3	ns	0.2879
	Source of Variation	F (DFn, DFd)	P
	Interaction	F (2, 43) = 3.207	0.0503
	Day	F (2, 43) = 17.84	<0.0001
	Sex	F (1, 22) = 4.410	0.0474
	post-hoc Sidak's multiple comp. test	Summary	Adjus. P
	Female, day 1 vs. day 2	ns	0.8453
	Female, day 1 vs. day 3	****	<0.0001
	Female, day 2 vs. day 3	***	0.0006
	Male, day 1 vs. day 2	*	0.0136
	Male, day 1 vs. day 3	**	0.0023
	Male, day 2 vs. day 3	ns	0.9004
	Female vs. Male, day 1	ns	0.2537
	Female vs. Male, day 2	#	0.0130
	Female vs. Male, day 3	ns	0.7167

Table 3 – Statistical analysis of data shown in Figure 4B for place learning, cohort A, first pass (6-8 weeks). A *p*-value less than 0.05 is considered significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001). ns = not significant.

Figure 4B	Mixed-effects analysis, place learning, cohort A, second pass		
Correct (C-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (2, 35) = 4.975	0.0125
	Day	F (2, 35) = 2.064	0.1422
	Sex	F (1, 20) = 0.2437	0.6269
Incorrect (IC-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (2, 35) = 5.580	0.0079
	Day	F (2, 35) = 6.305	0.0046
	Sex	F (1, 20) = 0.1981	0.6610
	post-hoc Sidak's multiple comp. test	Summary	Adjus. P
	Female, day 1 vs. day 2	ns	0.3829
	Female, day 1 vs. day 3	*	0.0470
	Female, day 2 vs. day 3	***	0.0009
	Male, day 1 vs. day 2	ns	0.0632
	Male, day 1 vs. day 3	ns	0.1004
	Male, day 2 vs. day 3	ns	0.9945

Table 4 – Statistical analysis of data shown in Figure 4B for place learning, cohort A, second pass (4-5 months). A *p*-value less than 0.05 is considered significant (**p* < 0.05, ****p* < 0.001). ns = not significant.

Figure 4C	Paired <i>t</i> tests, place learning, cohort A, pass comparison			
	Comparison	<i>t</i> , <i>df</i>	Summary	<i>P</i>
Correct (C-AUC)	Female, day 1	<i>t</i> =5.672, <i>df</i> =9	***	0.0003
	Male, day 1	<i>t</i> =1.141, <i>df</i> =9	ns	0.2834
	Female, day 2	<i>t</i> =4.655, <i>df</i> =9	**	0.0012
	Male, day 2	<i>t</i> =0.9056, <i>df</i> =9	ns	0.3888
	Female, day 3	<i>t</i> =5.332, <i>df</i> =9	***	0.0005
	Male, day 3	<i>t</i> =0.01567, <i>df</i> =10	ns	0.9878
Incorrect (IC-AUC)	Female, day 1	<i>t</i> =3.283, <i>df</i> =9	**	0.0095
	Male, day 1	<i>t</i> =1.055, <i>df</i> =9	ns	0.3189
	Female, day 2	<i>t</i> =0.5384, <i>df</i> =9	ns	0.6034
	Male, day 2	<i>t</i> =0.4251, <i>df</i> =9	ns	0.6807
	Female, day 3	<i>t</i> =0.7488, <i>df</i> =9	ns	0.4731
	Male, day 3	<i>t</i> =0.3990, <i>df</i> =10	ns	0.6983

Table 5 – Statistical analysis of data shown in Figure 4C for place learning, cohort A, pass comparison. A *p*-value less than 0.05 is considered significant (***p* < 0.01, ****p* < 0.001). ns = not significant.

258 ***App^{h/h}* rats in cohort A can acquire a place reversal task by 6-8 weeks of age with session-wise**
259 **improvement.** After place learning, rats in cohort A were started on the place reversal program,
260 which switches the correct corner in place learning to the one diagonally opposing it. Activity curves
261 showed qualitative improvement, like those shown for place learning (Figures 4A and 5A). There were
262 also significant session-wise increases in C-AUC and decreases in IC-AUC for both sexes during the
263 first pass, with no significant differences seen during the second pass for either sex (Figure 5B).
264 There were significant sex differences seen during the first pass, with C-AUC higher and IC-AUC
265 lower for males for every drinking session, but not during the second pass (Figure 5B). For females,
266 C-AUC was significantly higher during the second pass compared to the first for the 1st and 3rd
267 drinking sessions, with the value for the 2nd drinking session being higher but not reaching
268 significance, while IC-AUC was not significantly different for any drinking session; there were no

269 significant differences between passes for males (Figure 5C).

Figure 5 – Place reversal, cohort A. (A) Activity curves showing the fractional accumulation (y-axis) of visits over drinking session time (x-axis), reset every 180 minutes, by sex and pass. Curves for correct and incorrect visits are black and blue, respectively. **(B)** Sex comparison of area under the curve (AUC) for activity curves of individual animals by visit category and pass. “*” denotes significant comparisons within sex across program days, while “#” denotes those for the corresponding program day between sexes. Female (♀) and male (♂) data points are indicated by white and black circles, respectively. **(C)** Pass comparison of AUCs for activity curves of individual animals by sex, program day, and visit category. Data points from the first and second passes are indicated by white and red circles, respectively. All data represented as mean ± SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, similarly for # $p < 0.05$, etc.). See Tables 6-8 for statistical analysis. FP = first pass (6-8 weeks), SP = second pass (4-5 months), C = correct (visits), IC = incorrect (visits).

270

Figure 5B			
Two-way RM ANOVA, place reversal, cohort A, first pass			
Correct (C-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (2, 44) = 0.08855	0.9154
	Day	F (2, 44) = 12.26	<0.0001
	Sex	F (1, 22) = 15.38	0.0007
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female, day 1 vs. day 2	*	0.0277
	Female, day 1 vs. day 3	**	0.0020
	Female, day 2 vs. day 3	ns	0.7307
	Male, day 1 vs. day 2	ns	0.0713
	Male, day 1 vs. day 3	*	0.0109
	Male, day 2 vs. day 3	ns	0.8461
	Female vs. Male, day 1	##	0.0042
	Female vs. Male, day 2	##	0.0100
	Female vs. Male, day 3	#	0.0153
Incorrect (IC-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (2, 44) = 1.091	0.3449
	Day	F (2, 44) = 16.39	<0.0001
	Sex	F (1, 22) = 21.79	0.0001
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female, day 1 vs. day 2	**	0.0026
	Female, day 1 vs. day 3	****	<0.0001
	Female, day 2 vs. day 3	ns	0.5360
	Male, day 1 vs. day 2	ns	0.2942
	Male, day 1 vs. day 3	**	0.0083
	Male, day 2 vs. day 3	ns	0.3454
	Female vs. Male, day 1	###	0.0002
	Female vs. Male, day 2	#	0.0319
	Female vs. Male, day 3	#	0.0154

Table 6 – Statistical analysis of data shown in Figure 5B for place reversal, cohort A, first pass (6-8 weeks). A *p*-value less than 0.05 is considered significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001). ns = not significant.

Figure 5B			
Two-way RM ANOVA, place reversal, cohort A, second pass			
Correct (C-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (2, 38) = 0.4885	0.6174
	Day	F (2, 38) = 4.223	0.0221
	Sex	F (1, 19) = 0.02749	0.8701
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female, day 1 vs. day 2	ns	0.8887
	Female, day 1 vs. day 3	ns	0.2396
	Female, day 2 vs. day 3	ns	0.6212
	Male, day 1 vs. day 2	ns	0.1257
	Male, day 1 vs. day 3	ns	0.0804
Incorrect (IC-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (2, 38) = 0.01931	0.9809
	Day	F (2, 38) = 3.059	0.0586
	Sex	F (1, 19) = 0.01156	0.9155

Table 7 – Statistical analysis of data shown in Figure 5B for place reversal, cohort A, second pass (4-5 months). A *p*-value less than 0.05 is considered significant. ns = not significant.

Figure 5C	Paired <i>t</i> tests, place reversal, cohort A, pass comparison			
Correct (C-AUC)	Comparison	<i>t</i> , <i>df</i>	Summary	<i>P</i>
	Female, day 1	<i>t</i> =5.188, <i>df</i> =9	***	0.0006
	Male, day 1	<i>t</i> =0.06592, <i>df</i> =10	ns	0.9487
	Female, day 2	<i>t</i> =1.595, <i>df</i> =9	ns	0.1453
	Male, day 2	<i>t</i> =0.3032, <i>df</i> =10	ns	0.7680
	Female, day 3	<i>t</i> =3.329, <i>df</i> =9	**	0.0088
	Male, day 3	<i>t</i> =0.09445, <i>df</i> =10	ns	0.9266
Incorrect (IC-AUC)	Comparison	<i>t</i> , <i>df</i>	Summary	<i>P</i>
	Female, day 1	<i>t</i> =1.661, <i>df</i> =9	ns	0.1310
	Male, day 1	<i>t</i> =1.387, <i>df</i> =10	ns	0.1955
	Female, day 2	<i>t</i> =0.003776, <i>df</i> =9	ns	0.9971
	Male, day 2	<i>t</i> =1.032, <i>df</i> =10	ns	0.3265
	Female, day 3	<i>t</i> =0.6898, <i>df</i> =9	ns	0.5077
	Male, day 3	<i>t</i> =1.668, <i>df</i> =10	ns	0.1263

Table 8 – Statistical analysis of data shown in Figure 5C for place reversal, cohort A, pass comparison. A *p*-value less than 0.05 is considered significant (***p* < 0.01, ****p* < 0.001). ns = not significant.

272 **App^{h/h} rats in cohort B can acquire a place learning with corner switching task by 4-5 months**
273 **of age with session-wise improvement.** Rather than progressing from place learning to place
274 reversal as cohort A rats did, cohort B rats were started on the place learning with corner switch
275 program after single corner restriction. This program was designed to be a faster-paced combination
276 of place learning and place reversal, with correct corners switching every 45 minutes within a drinking
277 session. Activity curves show marked differences between passes for both sexes, with curves for
278 correct visits surpassing those for incorrect visits earlier during the second pass; notably, for females,
279 the correct curve surpassed the incorrect curve by the end of the 2nd drinking session during the
280 second pass, whereas the correct curve never surpassed the incorrect one during the first pass
281 (Figure 6A). No significant session-wise differences in AUC were seen during the first pass for either
282 sex, but there were significant increases in C-AUC and decreases in IC-AUC during the second pass
283 for both sexes, more pronounced for C-AUC (Figure 6B). Sex differences were significant for each
284 drinking session during the second pass, with C-AUC higher for females and IC-AUC lower for males
285 (Figure 6B). For females during the second pass compared to the first pass, C-AUC was significantly
286 higher for females for all but the 1st drinking session, while IC-AUC was only significantly different for
287 the 4th drinking session (Figure 6C). For males during the second pass compared to the first pass, C-
288 AUC was significantly higher for all drinking sessions, while IC-AUC was significantly lower for all

289 drinking sessions except the 2nd (Figure 6C).

Figure 6 – Place learning with corner switch, cohort B. (A) Activity curves showing the fractional accumulation (y-axis) of visits over drinking session time (x-axis), reset every 180 minutes, by sex and pass. Curves for correct and incorrect visits are black and blue, respectively. **(B)** Sex comparison of area under the curve (AUC) for activity curves of individual animals by visit category and pass. “*” denotes significant comparisons within sex across program days, while “#” denotes those for the corresponding program day between sexes. Female (♀) and male (♂) data points are indicated by white and black circles, respectively. **(C)** Pass comparison of AUCs for activity curves of individual animals by sex, program day, and visit category. Data points from the first and second passes are indicated by white and red circles, respectively. All data represented as mean ± SEM (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, similarly for #*p* < 0.05, etc.). See Tables 9-11 for statistical analysis. FP = first pass (6-8 weeks), SP = second pass (4-5 months), C = correct (visits), IC = incorrect (visits).

290

Figure 6B	Two-way RM ANOVA, place learning with corner switch, cohort B, first pass		
Correct (C-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 63) = 1.196	0.3186
	Day	F (3, 63) = 0.3236	0.8083
	Sex	F (1, 21) = 2.476	0.1306
Incorrect (IC-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 63) = 0.6067	0.6131
	Day	F (3, 63) = 1.309	0.2795
	Sex	F (1, 21) = 4.759	0.0406
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female vs. Male, day 1	ns	0.9552
	Female vs. Male, day 2	ns	0.1174
	Female vs. Male, day 3	ns	0.6359
	Female vs. Male, day 4	ns	0.9713

Table 9 – Statistical analysis of data shown in Figure 6B for place learning with corner switch, cohort B, first pass (6-8 weeks). A *p*-value less than 0.05 is considered significant. ns = not significant.

Figure 6B	Two-way RM ANOVA, place learning with corner switch, cohort B, second pass		
Correct (C-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 60) = 1.594	0.2003
	Day	F (3, 60) = 13.62	<0.0001
	Sex	F (1, 20) = 68.24	<0.0001
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female, day 1 vs. day 4	**	0.0011
	Female, day 2 vs. day 4	**	0.0039
	Female, day 3 vs. day 4	**	0.0013
	Male, day 1 vs. day 2	ns	0.8863
	Male, day 1 vs. day 3	*	0.0324
	Male, day 1 vs. day 4	***	0.0002
	Male, day 2 vs. day 3	ns	0.3527
	Male, day 2 vs. day 4	**	0.0068
	Male, day 3 vs. day 4	ns	0.5392
	Female vs. Male, day 1	###	0.0001
	Female vs. Male, day 2	####	<0.0001
	Female vs. Male, day 3	####	<0.0001
	Female vs. Male, day 4	####	<0.0001
Incorrect (IC-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 60) = 1.427	0.2438
	Day	F (3, 60) = 7.773	0.0002
	Sex	F (1, 20) = 39.84	<0.0001
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female, day 1 vs. day 4	ns	0.0821
	Female, day 2 vs. day 4	*	0.0423
	Female, day 3 vs. day 4	ns	0.0773
	Male, day 1 vs. day 2	ns	0.4405
	Male, day 1 vs. day 3	ns	0.5832
	Male, day 1 vs. day 4	ns	0.1874
	Male, day 2 vs. day 3	*	0.0122
	Male, day 2 vs. day 4	**	0.0016
	Female vs. Male, day 1	###	0.0006
	Female vs. Male, day 2	#	0.0279
	Female vs. Male, day 3	####	<0.0001
	Female vs. Male, day 4	##	0.0032

Table 10 – Statistical analysis of data shown in Figure 6B for place learning with corner switch, cohort B, second pass (4-5 months). A p -value less than 0.05 is considered significant (* p < 0.05, ** p < 0.01, *** p < 0.001, #### p < 0.0001). Some non-significant comparisons are omitted. ns = not significant.

Figure 6C		Paired <i>t</i> tests, place learning with corner switch, cohort B, pass comparison		
Correct (C-AUC)	Comparison	<i>t</i> , <i>df</i>	Summary	<i>P</i>
	Female, day 1	<i>t</i> =1.200, <i>df</i> =9	ns	0.2606
	Male, day 1	<i>t</i> =3.645, <i>df</i> =11	**	0.0039
	Female, day 2	<i>t</i> =2.577, <i>df</i> =9	*	0.0298
	Male, day 2	<i>t</i> =5.053, <i>df</i> =11	***	0.0004
	Female, day 3	<i>t</i> =3.192, <i>df</i> =9	*	0.0110
	Male, day 3	<i>t</i> =6.174, <i>df</i> =11	****	<0.0001
	Female, day 4	<i>t</i> =4.269, <i>df</i> =9	**	0.0021
	Male, day 4	<i>t</i> =9.086, <i>df</i> =11	****	<0.0001
Incorrect (IC-AUC)	Comparison	<i>t</i> , <i>df</i>	Summary	<i>P</i>
	Female, day 1	<i>t</i> =0.2539, <i>df</i> =9	ns	0.8053
	Male, day 1	<i>t</i> =2.684, <i>df</i> =11	*	0.0213
	Female, day 2	<i>t</i> =0.7319, <i>df</i> =9	ns	0.4828
	Male, day 2	<i>t</i> =0.8458, <i>df</i> =11	ns	0.4157
	Female, day 3	<i>t</i> =0.6925, <i>df</i> =9	ns	0.5061
	Male, day 3	<i>t</i> =3.845, <i>df</i> =11	**	0.0027
	Female, day 4	<i>t</i> =2.893, <i>df</i> =9	*	0.0178
	Male, day 4	<i>t</i> =4.633, <i>df</i> =11	***	0.0007

Table 11 – Statistical analysis of data shown in Figure 6C for place learning with corner switch, cohort B, pass comparison. A *p*-value less than 0.05 is considered significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001). ns = not significant.

App^{h/h} rats in cohorts A and B can acquire a behavioral sequencing task by 6-8 weeks of age with session-wise improvement.

After place learning and place reversal (cohort A) or place learning with corner switch (cohort B), we further tested the rats' spatial learning capabilities with a behavioral sequencing program requiring the animals to shuttle between diagonally opposing corners for water access. Visits were categorized as correct (C), lateral (L), or opposite (O) as described in the methods, with activity curves generated (Figures 7A and 8A) and AUC analysis performed (Figures 7B-C and 8B-C) similarly as in other programs. Cohort A rats of both sexes during the first pass showed significant increases in C-AUC and decreases in O-AUC, but no significant changes in L-AUC (Figure 7B). These changes were consistent during the second pass for females, whereas males no longer showed significant session-wise changes in C-AUC (Figure 7B). There were significant sex differences observed for the 3rd drinking session of the first pass for C-AUC (higher in males) and L-AUC (higher in females), with no significant differences observed during the second pass (Figure 7B). For females during the second pass compared to the first, C-AUC was significantly greater for the 1st drinking session; for males, C-AUC was lower while L-AUC was higher for the 3rd drinking session (Figure 7C). Cohort B rats

307 exhibited a different activity profile: for females, the session-wise differences in C-AUC, L-AUC, and
 308 O-AUC were minimal during both passes, whereas for males, there were many significant session-
 309 wise differences, especially during the second pass with increases in C-AUC and decreases in L-AUC
 310 and O-AUC (Figure 8B). Significant differences found between passes for females were sporadic for
 311 C-AUC (1st drinking session), L-AUC (4th drinking session), and O-AUC (1st and 3rd drinking sessions);
 312 in contrast, for males, C-AUC was significantly higher for every drinking session during the second
 313 pass compared to the first, with L-AUC lower for every drinking session except the 1st and O-AUC
 314 higher in the 1st and 2nd drinking sessions (Figure 8C).

Figure 7 – Behavioral sequencing, cohort A. (A) Activity curves showing the fractional accumulation (y-axis) of visits over drinking session time (x-axis), reset every 180 minutes, by sex and pass. Curves for correct, lateral, and opposite visits are black, blue, and red, respectively. (B) Sex comparison of area under the curve (AUC) for activity curves of individual animals by visit category and pass. “*” denotes significant comparisons within sex across program days, while “#” denotes those for the corresponding program day between sexes. Female (♀) and male (♂) data points are indicated by white and black circles, respectively. (C) Pass comparison of AUCs for activity curves of individual animals by sex, program day, and visit category. Data points from the first and second passes are indicated by white and red circles, respectively. All data are represented as mean ± SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, similarly for # $p < 0.05$, etc.). See Tables 12-14 for statistical analysis. FP = first pass (6-8 weeks), SP = second pass (4-5 months), C = correct (visits), L = lateral (visits), O = opposite (visits).

Figure 8 – Behavioral sequencing, cohort B. (A) Activity curves showing the fractional accumulation (y-axis) of visits over drinking session time (x-axis), reset every 180 minutes, by sex and pass. Curves for correct, lateral, and opposite visits are black, blue, and red, respectively. (B) Sex comparison of area under the curve (AUC) for activity curves of individual animals by visit category and pass. “*” denotes significant comparisons within sex across program days, while “#” denotes those for the corresponding program day between sexes. Female (♀) and male (♂) data points are indicated by white and black circles, respectively. (C) Pass comparison of AUCs for activity curves of individual animals by sex, program day, and visit category. Data points from the first and second passes are indicated by white and red circles, respectively. All data are represented as mean ± SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, similarly for # $p < 0.05$, etc.). See Tables 15-17 for statistical analysis. FP = first pass (6-8 weeks), SP = second pass (4-5 months), C = correct (visits), L = lateral (visits), O = opposite (visits).

Figure 7B			
Two-way RM ANOVA, behavioral sequencing, cohort A, first pass			
Correct (C-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (2, 44) = 3.323	0.0453
	Day	F (2, 44) = 22.41	<0.0001
	Sex	F (1, 22) = 2.906	0.1023
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female, day 1 vs. day 2	ns	0.2721
	Female, day 1 vs. day 3	*	0.0168
	Female, day 2 vs. day 3	ns	0.5330
	Male, day 1 vs. day 2	***	0.0004
	Male, day 1 vs. day 3	****	<0.0001
	Male, day 2 vs. day 3	ns	0.0775
Lateral (L-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (2, 44) = 1.391	0.2595
	Day	F (2, 44) = 0.6175	0.5439
	Sex	F (1, 22) = 7.170	0.0137
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female vs. Male, day 1	ns	0.2961
	Female vs. Male, day 2	ns	0.0578
	Female vs. Male, day 3	#	0.0109
Opposite (O-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (2, 44) = 0.09638	0.9083
	Day	F (2, 44) = 15.98	<0.0001
	Sex	F (1, 22) = 2.116	0.1599
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female, day 1 vs. day 2	**	0.0055
	Female, day 1 vs. day 3	**	0.0039
	Female, day 2 vs. day 3	ns	0.9992
	Male, day 1 vs. day 2	**	0.0088
	Male, day 1 vs. day 3	**	0.0011
	Male, day 2 vs. day 3	ns	0.8555

Table 12 – Statistical analysis of data shown in Figure 7B for behavioral sequencing, cohort A, first pass (6-8 weeks). A *p*-value less than 0.05 is considered significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001). ns = not significant.

318	Figure 7B	Two-way RM ANOVA, behavioral sequencing, cohort A, second pass		
	Correct (C-AUC)	Source of Variation	F (DFn, DFd)	P
319		Interaction	F (2, 38) = 1.537	0.2281
		Day	F (2, 38) = 5.618	0.0073
		Sex	F (1, 19) = 3.569	0.0742
320		post-hoc Sidak's multiple comp. test	Summary	Adjusted P
		Female, day 1 vs. day 2	*	0.0474
321		Female, day 1 vs. day 3	*	0.0278
		Female, day 2 vs. day 3	ns	0.9950
322		Male, day 1 vs. day 2	ns	0.9988
		Male, day 1 vs. day 3	ns	0.1598
		Male, day 2 vs. day 3	ns	0.2075
323	Lateral (L-AUC)	Source of Variation	F (DFn, DFd)	P
		Interaction	F (2, 38) = 0.07518	0.9277
324		Day	F (2, 38) = 0.4424	0.6458
		Sex	F (1, 19) = 0.5818	0.4550
325	Opposite (O-AUC)	Source of Variation	F (DFn, DFd)	P
		Interaction	F (2, 38) = 0.7340	0.4867
		Day	F (2, 38) = 12.12	<0.0001
		Sex	F (1, 19) = 0.00986	0.9219
326		post-hoc Sidak's multiple comp. test	Summary	Adjusted P
		Female, day 1 vs. day 2	*	0.0395
327		Female, day 1 vs. day 3	***	0.0006
		Female, day 2 vs. day 3	ns	0.3448
328		Male, day 1 vs. day 2	ns	0.1123
		Male, day 1 vs. day 3	*	0.0379
		Male, day 2 vs. day 3	ns	0.9526

Table 13 – Statistical analysis of data shown in Figure 7B for behavioral sequencing, cohort A, second pass (4-5 months). A *p*-value less than 0.05 is considered significant (**p* < 0.05, ****p* < 0.001). ns = not significant.

Figure 7C	Paired <i>t</i> tests, behavioral sequencing, cohort A, pass comparison			
	Comparison	t, df	Summary	P
Correct (C-AUC)	Female, day 1	t=2.491, df=8	*	0.0375
	Male, day 1	t=0.2282, df=11	ns	0.8237
	Female, day 2	t=1.032, df=8	ns	0.3324
	Male, day 2	t=1.474, df=11	ns	0.1686
	Female, day 3	t=1.177, df=8	ns	0.2728
	Male, day 3	t=1.844, df=11	ns	0.0923
Lateral (L-AUC)	Comparison	t, df	Summary	P
	Female, day 1	t=0.9930, df=8	ns	0.3498
	Male, day 1	t=1.502, df=11	ns	0.1613
	Female, day 2	t=1.498, df=8	ns	0.1724
	Male, day 2	t=2.034, df=11	ns	0.0668
	Female, day 3	t=0.3042, df=8	ns	0.7687
	Male, day 3	t=3.328, df=11	**	0.0067
Opposite (O-AUC)	Comparison	t, df	Summary	P
	Female, day 1	t=0.9581, df=8	ns	0.3661
	Male, day 1	t=0.9490, df=11	ns	0.3630
	Female, day 2	t=0.6758, df=8	ns	0.5182
	Male, day 2	t=1.496, df=11	ns	0.1628
	Female, day 3	t=0.4350, df=8	ns	0.6751
	Male, day 3	t=0.7209, df=11	ns	0.4860

Table 14 – Statistical analysis of data shown in Figure 7C for behavioral sequencing, cohort A, pass comparison. A *p*-value less than 0.05 is considered significant (**p* < 0.05, ***p* < 0.01). ns = not significant.

Figure 8B	Mixed-effects analysis, behavioral sequencing, cohort B, first pass		
Correct (C-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (4, 77) = 1.576	0.1891
	Day	F (4, 77) = 6.336	0.0002
	Sex	F (1, 21) = 0.5258	0.4764
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female, day 1 vs. day 2	ns	>0.9999
	Female, day 1 vs. day 3	ns	>0.9999
	Female, day 1 vs. day 4	ns	0.9681
	Female, day 1 vs. day 5	ns	0.5746
	Female, day 2 vs. day 3	ns	>0.9999
	Female, day 2 vs. day 4	ns	0.9994
	Female, day 2 vs. day 5	ns	0.8721
	Female, day 3 vs. day 4	ns	0.9980
	Female, day 3 vs. day 5	ns	0.8138
	Female, day 4 vs. day 5	ns	0.9987
	Male, day 1 vs. day 2	ns	0.6038
	Male, day 1 vs. day 3	ns	0.0745
	Male, day 1 vs. day 4	***	0.0004
	Male, day 1 vs. day 5	***	0.0002
	Male, day 2 vs. day 3	ns	0.9644
	Male, day 2 vs. day 4	ns	0.0898
	Male, day 2 vs. day 5	*	0.0388
	Male, day 3 vs. day 4	ns	0.7651
	Male, day 3 vs. day 5	ns	0.4960
	Male, day 4 vs. day 5	ns	>0.9999
Lateral (L-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (4, 77) = 1.215	0.3114
	Day	F (4, 77) = 1.408	0.2394
	Sex	F (1, 21) = 0.0001	0.9910
Opposite (O-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (4, 77) = 0.7529	0.5592
	Day	F (4, 77) = 0.4977	0.7374
	Sex	F (1, 21) = 0.0369	0.8496

Table 15 – Statistical analysis of data shown in Figure 8B for behavioral sequencing, cohort B, first pass (6-8 weeks). A *p*-value less than 0.05 is considered significant (**p* < 0.05, ****p* < 0.001). ns = not significant.

Figure 8B	Two-way RM ANOVA, behavioral sequencing, cohort B, second pass		
Correct (C-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (4, 76) = 32.02	<0.0001
	Day	F (4, 76) = 46.43	<0.0001
	Sex	F (1, 19) = 84.69	<0.0001
	post-hoc Sidak's multiple comparisons test	Summary	Adjusted P
	Male, day 1 vs. days 2, 3, 4, 5	****	<0.0001
	Male, day 2 vs. day 3	****	<0.0001
	Male, day 2 vs. day 4	****	<0.0001
	Male, day 2 vs. day 5	****	<0.0001
	Female vs Male, day 2	####	<0.0001
	Female vs Male, day 3	####	<0.0001
	Female vs Male, day 4	####	<0.0001
	Female vs Male, day 5	####	<0.0001
Lateral (L-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (4, 76) = 3.906	0.0061
	Day	F (4, 76) = 7.183	<0.0001
	Sex	F (1, 19) = 13.76	0.0015
	post-hoc Sidak's multiple comparisons test	Summary	Adjusted P
	Female, day 4 vs. day 5	*	0.0155
	Male, day 1 vs. day 3	***	0.0006
	Male, day 1 vs. day 4	***	0.0001
	Male, day 1 vs. day 5	***	0.0001
	Female vs. male, day 3	##	0.0011
	Female vs. male, day 4	####	<0.0001
Opposite (O-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (4, 76) = 3.607	0.0096
	Day	F (4, 76) = 13.74	<0.0001
	Sex	F (1, 19) = 0.1984	0.6611
	post-hoc Sidak's multiple comparisons test	Summary	Adjusted P
	Male, day 1 vs. day 2	*	0.0227
	Male, day 1 vs. day 3	***	0.0004
	Male, day 1 vs. day 4	****	<0.0001
	Male, day 1 vs. day 5	****	<0.0001
	Male, day 2 vs. day 4	*	0.0307
	Male, day 2 vs. day 5	**	0.0014

Table 16 – Statistical analysis of data shown in Figure 8B for behavioral sequencing, cohort B, second pass (4-5 months). A *p*-value less than 0.05 is considered significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, ****/####*p* < 0.0001). Non-significant comparisons have been omitted.

Figure 8C	Paired t tests, behavioral sequencing, cohort B, pass comparison			
Correct (C-AUC)	Comparison	t, df	Summary	P
	Female, day 1	t=2.393, df=9	*	0.0403
	Male, day 1	t=2.266, df=9	*	0.0497
	Female, day 2	t=0.8613, df=9	ns	0.4114
	Male, day 2	t=4.899, df=9	***	0.0008
	Female, day 3	t=0.8399, df=9	ns	0.4227
	Male, day 3	t=10.14, df=9	****	<0.0001
	Female, day 4	t=1.226, df=9	ns	0.2512
	Male, day 4	t=7.283, df=9	****	<0.0001
	Female, day 5	t=1.809, df=9	ns	0.1039
	Male, day 5	t=7.034, df=9	****	<0.0001
Lateral (L-AUC)	Comparison	t, df	Summary	P
	Female, day 1	t=1.466, df=9	ns	0.1768
	Male, day 1	t=2.158, df=9	ns	0.0593
	Female, day 2	t=0.6829, df=9	ns	0.5118
	Male, day 2	t=2.293, df=9	*	0.0475
	Female, day 3	t=0.4833, df=9	ns	0.6404
	Male, day 3	t=3.887, df=9	**	0.0037
	Female, day 4	t=2.374, df=9	*	0.0416
	Male, day 4	t=3.201, df=9	*	0.0108
	Female, day 5	t=0.5710, df=9	ns	0.5820
	Male, day 5	t=3.066, df=9	*	0.0134
Opposite (O-AUC)	Comparison	t, df	Summary	P
	Female, day 1	t=3.144, df=9	*	0.0119
	Male, day 1	t=3.901, df=9	**	0.0036
	Female, day 2	t=1.403, df=9	ns	0.1942
	Male, day 2	t=3.349, df=9	**	0.0085
	Female, day 3	t=2.459, df=9	*	0.0362
	Male, day 3	t=1.407, df=9	ns	0.1931
	Female, day 4	t=0.4780, df=9	ns	0.6441
	Male, day 4	t=0.6623, df=9	ns	0.5244
	Female, day 5	t=0.08419, df=9	ns	0.9347
	Male, day 5	t=0.5445, df=9	ns	0.5993

Table 17 – Statistical analysis of data shown in Figure 8C for behavioral sequencing, cohort B, pass comparison. A *p*-value less than 0.05 is considered significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001). ns = not significant.

App^{h/h} rats in cohort A and B may not be able to acquire a serial reversal task by 4-5 months of age.

335 We ended the timeline for both cohorts with a serial reversal program designed to add a layer of
 336 complexity to behavioral sequencing by requiring the rats to alternate diagonals after every eight
 337 correct nosepokes. For cohort A, qualitatively, activity curves for both sexes did not show much
 338 difference between passes or session-wise improvement (Figure 9A). Session-wise differences in
 339 AUC were minimal for both sexes during both passes, with some significant sex differences during the
 340 second pass (Figure 9B). Significant differences between passes were sporadic for females in L-AUC
 341 (2nd drinking session) and O-AUC (1st drinking session), and non-existent for males (Figure 9C). For
 342 cohort B, the activity curves show a possible difference between the first and second pass for males,
 343 but no session-wise differences (Figure 10A). AUC analysis revealed that for males compared to
 344 females during the second pass, C-AUC was significantly higher for all drinking sessions, with O-AUC
 345 significantly lower for the 3rd and 4th drinking sessions (Figure 10B). For males during the second pass
 346 compared to the first, C-AUC was significantly higher for every drinking session, with L-AUC
 347 significantly higher for the 4th drinking session; significant differences between passes were non-
 348 existent for females (Figure 10C).

Figure 9 – Serial reversal, cohort A. (A) Activity curves showing the fractional accumulation (y-axis) of visits over drinking session time (x-axis), reset every 180 minutes, by sex and pass. Curves for correct, lateral, and opposite visits are black, blue, and red, respectively. **(B)** Sex comparison of area under the curve (AUC) for activity curves of individual animals by visit category and pass. “*” denotes significant comparisons within sex across program days, while “#” denotes those for the corresponding program day between sexes. Female (♀) and male (♂) data points are indicated by white and black circles, respectively. **(C)** Pass comparison of AUCs for activity curves of individual animals by sex, program day, and visit category. Data points from the first and second passes are indicated by white and red circles, respectively. All data are represented as mean ± SEM (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, similarly for #*p* < 0.05, etc.). See Tables 18-20 for statistical analysis. FP = first pass (6-8 weeks), SP = second pass (4-5 months), C = correct (visits), L = lateral (visits), O = opposite (visits).

Figure 10 – Serial reversal, cohort B. (A) Activity curves showing the fractional accumulation (y-axis) of visits over drinking session time (x-axis), reset every 180 minutes, by sex and pass. Curves for correct, lateral, and opposite visits are black, blue, and red, respectively. **(B)** Sex comparison of area under the curve (AUC) for activity curves of individual animals by visit category and pass. “*” denotes significant comparisons within sex across program days, while “#” denotes those for the corresponding program day between sexes. Female (♀) and male (♂) data points are indicated by white and black circles, respectively. **(C)** Pass comparison of AUCs for activity curves of individual animals by sex, program day and visit category. Data points from the first and second passes are indicated by white and red circles, respectively. All data are represented as mean ± SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, similarly for # $p < 0.05$, etc.). See Tables 21-23 for statistical analysis. FP = first pass (6-8 weeks), SP = second pass (4-5 months), C = correct (visits), L = lateral (visits), O = opposite (visits).

Figure 9B			
Two-way RM ANOVA, serial reversal, cohort A, first pass			
Correct (C-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 66) = 0.3061	0.8209
	Day	F (3, 66) = 0.4277	0.7338
	Sex	F (1, 22) = 0.5099	0.4827
Lateral (L-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 66) = 0.9907	0.4027
	Day	F (3, 66) = 0.5091	0.6774
	Sex	F (1, 22) = 0.00011	0.9915
Opposite (O-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 66) = 0.4999	0.6837
	Day	F (3, 66) = 2.051	0.1152
	Sex	F (1, 22) = 0.0628	0.8044

Table 18 – Statistical analysis of data shown in Figure 9B for serial reversal, cohort A, first pass (6-8 weeks). A p -value less than 0.05 is considered significant.

Figure 9B	Mixed-effects analysis, serial reversal, cohort A, second pass		
Correct (C-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 53) = 0.6830	0.5664
	Day	F (3, 53) = 2.568	0.0641
	Sex	F (1, 19) = 5.754	0.0269
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female vs. Male, day 1	ns	0.8521
	Female vs. Male, day 2	ns	0.1465
	Female vs. Male, day 3	ns	0.1190
	Female vs. Male, day 4	ns	0.1574
Lateral (L-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 53) = 1.923	0.1370
	Day	F (3, 53) = 0.5768	0.6328
	Sex	F (1, 19) = 10.73	0.0040
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female vs. male, day 1	ns	0.9988
	Female vs. male, day 2	#	0.0228
	Female vs. male, day 3	#	0.0203
	Female vs. male, day 4	ns	0.8612
Opposite (O-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 53) = 2.117	0.1090
	Day	F (3, 53) = 1.178	0.3271
	Sex	F (1, 19) = 1.600	0.2212

Table 19 – Statistical analysis of data shown in Figure 9B for serial reversal, cohort A, second pass (4-5 months). A *p*-value less than 0.05 is considered significant (*#p* < 0.05). ns = not significant.

Figure 9C				
Paired <i>t</i> tests, serial reversal, cohort A, pass comparison				
Correct (C-AUC)	Comparison	t, df	Summary	P
	Female, day 1	t=0.1544, df=9	ns	0.8807
	Male, day 1	t=0.0315, df=9	ns	0.9756
	Female, day 2	t=0.1535, df=9	ns	0.8814
	Male, day 2	t=1.396, df=11	ns	0.1902
	Female, day 3	t=0.6986, df=9	ns	0.5024
	Male, day 3	t=0.8514, df=8	ns	0.4193
	Female, day 4	t=0.2108, df=9	ns	0.8377
	Male, day 4	t=0.9781, df=9	ns	0.3536
Lateral (L-AUC)	Comparison	t, df	Summary	P
	Female, day 1	t=0.3688, df=9	ns	0.7208
	Male, day 1	t=0.1354, df=9	ns	0.8952
	Female, day 2	t=4.072, df=9	**	0.0028
	Male, day 2	t=1.438, df=10	ns	0.1809
	Female, day 3	t=1.231, df=9	ns	0.2496
	Male, day 3	t=1.156, df=8	ns	0.2809
	Female, day 4	t=0.6974, df=9	ns	0.5031
	Male, day 4	t=1.581, df=9	ns	0.1483
Opposite (O-AUC)	Comparison	t, df	Summary	P
	Female, day 1	t=2.629, df=9	*	0.0274
	Male, day 1	t=0.2406, df=10	ns	0.8147
	Female, day 2	t=0.8891, df=9	ns	0.3971
	Male, day 2	t=0.1268, df=10	ns	0.9016
	Female, day 3	t=0.6236, df=9	ns	0.5483
	Male, day 3	t=0.1706, df=8	ns	0.8688
	Female, day 4	t=0.7629, df=9	ns	0.4651
	Male, day 4	t=0.4479, df=9	ns	0.6648

Table 20 – Statistical analysis of data shown in Figure 7C for serial reversal, cohort A, pass comparison. A *p*-value less than 0.05 is considered significant (**p* < 0.05, ***p* < 0.01). ns = not significant.

Figure 10B	Mixed-effects analysis, serial reversal, cohort B, first pass		
	Source of Variation	F (DFn, DFd)	P
Correct (C-AUC)	Interaction	F (3, 52) = 1.150	0.3378
	Day	F (3, 52) = 0.3990	0.7542
	Sex	F (1, 19) = 0.8525	0.3674
Lateral (L-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 52) = 0.9508	0.4229
	Day	F (3, 52) = 0.5477	0.6519
Opposite (O-AUC)	Sex	F (1, 19) = 0.0230	0.8811
	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 52) = 0.1502	0.9291
	Day	F (3, 52) = 0.7863	0.5070
	Sex	F (1, 19) = 1.071	0.3136

Table 21 – Statistical analysis of data shown in Figure 10B for serial reversal, cohort B, first pass (6-8 weeks). A *p*-value less than 0.05 is considered significant.

Figure 10B	Two-way RM ANOVA, serial reversal, cohort B, second pass		
	Source of Variation	F (DFn, DFd)	P
Correct (C-AUC)	Interaction	F (3, 57) = 1.357	0.2652
	Day	F (3, 57) = 1.391	0.2548
	Sex	F (1, 19) = 69.20	<0.0001
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female vs. Male, day 1	####	<0.0001
	Female vs. Male, day 2	####	<0.0001
	Female vs. Male, day 3	####	<0.0001
	Female vs. Male, day 4	####	<0.0001
Lateral (L-AUC)	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 57) = 0.0096	0.9987
	Day	F (3, 57) = 0.1552	0.9259
Opposite (O-AUC)	Sex	F (1, 19) = 2.888	0.1056
	Source of Variation	F (DFn, DFd)	P
	Interaction	F (3, 57) = 0.6388	0.5932
	Day	F (3, 57) = 0.0676	0.9769
	Sex	F (1, 19) = 20.49	0.0002
	post-hoc Sidak's multiple comp. test	Summary	Adjusted P
	Female vs. Male, day 1	ns	0.2627
	Female vs. Male, day 2	ns	0.1193
	Female vs. Male, day 3	#	0.0146
	Female vs. Male, day 4	##	0.0034

Table 22 – Statistical analysis of data shown in Figure 10B for serial reversal, cohort B, second pass (4-5 months). A *p*-value less than 0.05 is considered significant (**p* < 0.05, ***p* < 0.01, *****p* < 0.0001). ns = not significant.

Figure 10C	Paired t tests, serial reversal, cohort B, pass comparison			
Correct (C-AUC)	Comparison	t, df	Summary	P
	Female, day 1	t=0.8066, df=9	ns	0.4407
	Male, day 1	t=5.117, df=9	***	0.0006
	Female, day 2	t=0.5204, df=9	ns	0.6153
	Male, day 2	t=5.011, df=8	**	0.0010
	Female, day 3	t=0.4795, df=9	ns	0.6430
	Male, day 3	t=5.852, df=7	***	0.0006
	Female, day 4	t=0.7748, df=9	ns	0.4583
	Male, day 4	t=3.116, df=7	*	0.0169
Lateral (L-AUC)	Comparison	t, df	Summary	P
	Female, day 1	t=0.4160, df=9	ns	0.6872
	Male, day 1	t=1.975, df=9	ns	0.0797
	Female, day 2	t=0.7012, df=9	ns	0.5009
	Male, day 2	t=0.5277, df=8	ns	0.6120
	Female, day 3	t=0.5298, df=9	ns	0.6091
	Male, day 3	t=0.7421, df=7	ns	0.4822
	Female, day 4	t=0.4336, df=9	ns	0.6748
	Male, day 4	t=4.836, df=7	**	0.0019
Opposite (O-AUC)	Comparison	t, df	Summary	P
	Female, day 1	t=0.6327, df=9	ns	0.5427
	Male, day 1	t=2.133, df=9	ns	0.0617
	Female, day 2	t=0.3549, df=9	ns	0.7308
	Male, day 2	t=1.153, df=8	ns	0.2821
	Female, day 3	t=0.3089, df=9	ns	0.7644
	Male, day 3	t=1.405, df=7	ns	0.2028
	Female, day 4	t=0.0202, df=9	ns	0.9843
	Male, day 4	t=2.342, df=7	ns	0.0517

Table 23 – Statistical analysis of data shown in Figure 10C for serial reversal, cohort B, pass comparison. A *p*-value less than 0.05 is considered significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). ns = not significant.

356

357 Discussion

358 *App^{h/h}* rats of both sexes were able to adapt to the IntelliCage and acquire simple place learning and
 359 reversal tasks, as well as a more complex behavioral sequencing task, by 6-8 weeks of age. Males
 360 tended to perform better than females at 4-5 months of age in place learning with corner switch and
 361 behavioral sequencing. The results of the single corner restriction program for cohort B suggest that
 362 although individual variance exists among the rats, it is small enough that animals can be

363 approximated as identical subjects for these IntelliCage experiments. Generating activity curves with
364 aggregate cohort data is one way to reduce the impact of this variance on interpretation of cohort
365 performance. Using AUC as a metric for comparing activity between groups is a natural extension of
366 using linear fits on activity curves to estimate learning rate and takes full advantage of the data
367 volume the IntelliCage offers. Task acquisition can be characterized by performance parameters—in
368 this case, AUC—that are greater or less than the value that would be expected through chance alone,
369 depending on the visit category. Chance C-AUC/O-AUC would be equal to the area of a right triangle
370 with base of length 180 (number of minutes in a drinking session) and height of 0.25 (probability of
371 visiting a correct/opposite corner at random), or 22.5. Similar calculations can be done for IC-AUC
372 ($180 \times 0.75 = 67.5$) and L-AUC ($180 \times 0.50 = 90$). Significant session-wise differences in the
373 appropriate direction can reflect task acquisition too, as seen with increases in C-AUC accompanied
374 by decreases in IC-AUC, L-AUC, or O-AUC. These characteristics were observed for all the spatial
375 learning programs except serial reversal, suggesting that the program is too complex for the rats to
376 learn by 4-5 months of age. In general, a task that challenges the animals without being impossible to
377 acquire would be ideal for identifying possible cognitive deficits in models of neurodegeneration and
378 dementia. Task acquisition of behavioral sequencing but not serial reversal suggests that a program
379 of intermediate difficulty using a sequence involving all four corners (in clockwise motion, for example)
380 rather than just two in a single diagonal, might be worth testing in future studies. By these measures,
381 this study establishes a baseline spatial learning profile for *App^{h/h}* control rats while providing initial
382 validation of analytic methods exploring aggregate cohort activity and using AUC as a metric for task
383 performance in the IntelliCage.

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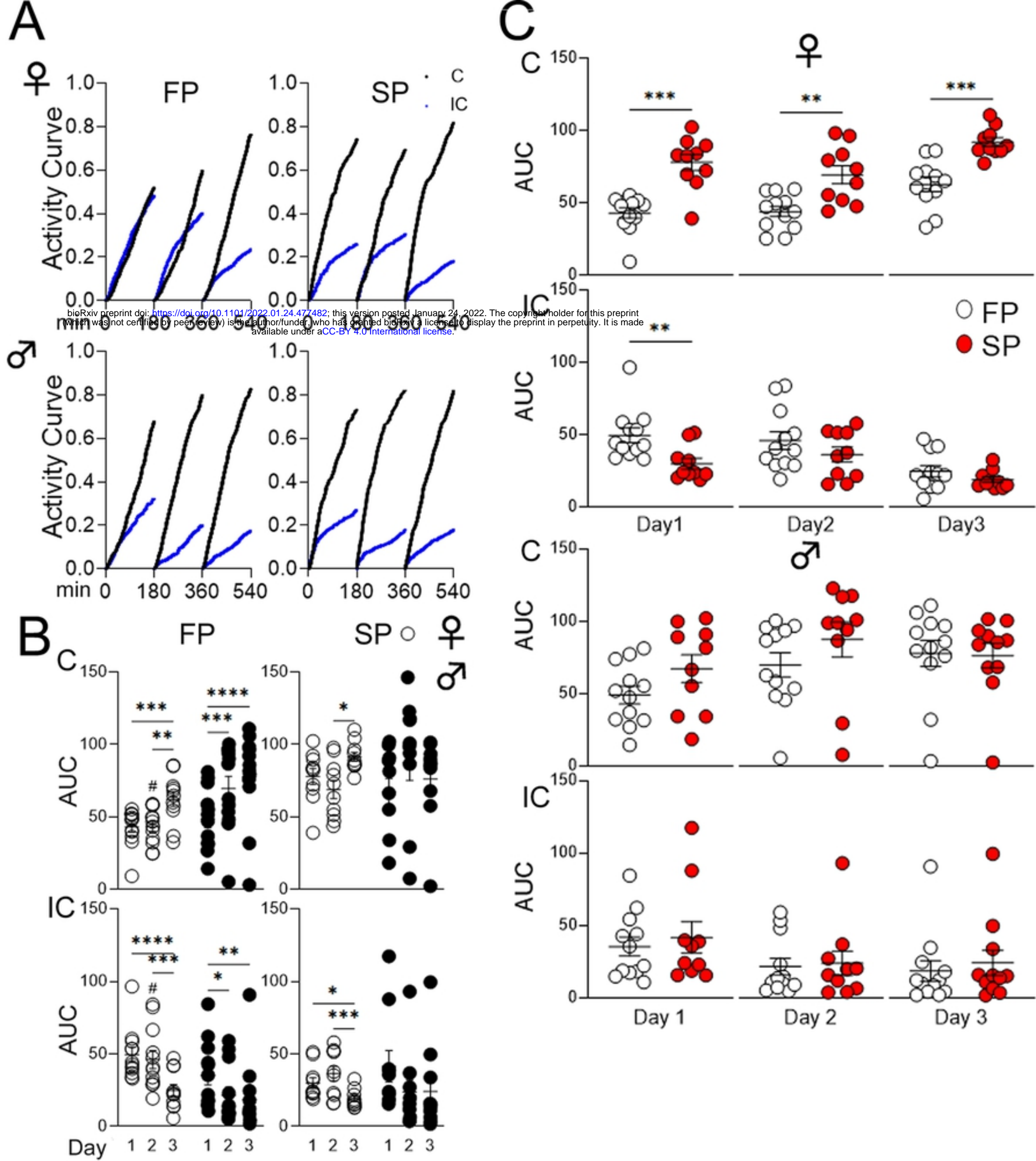
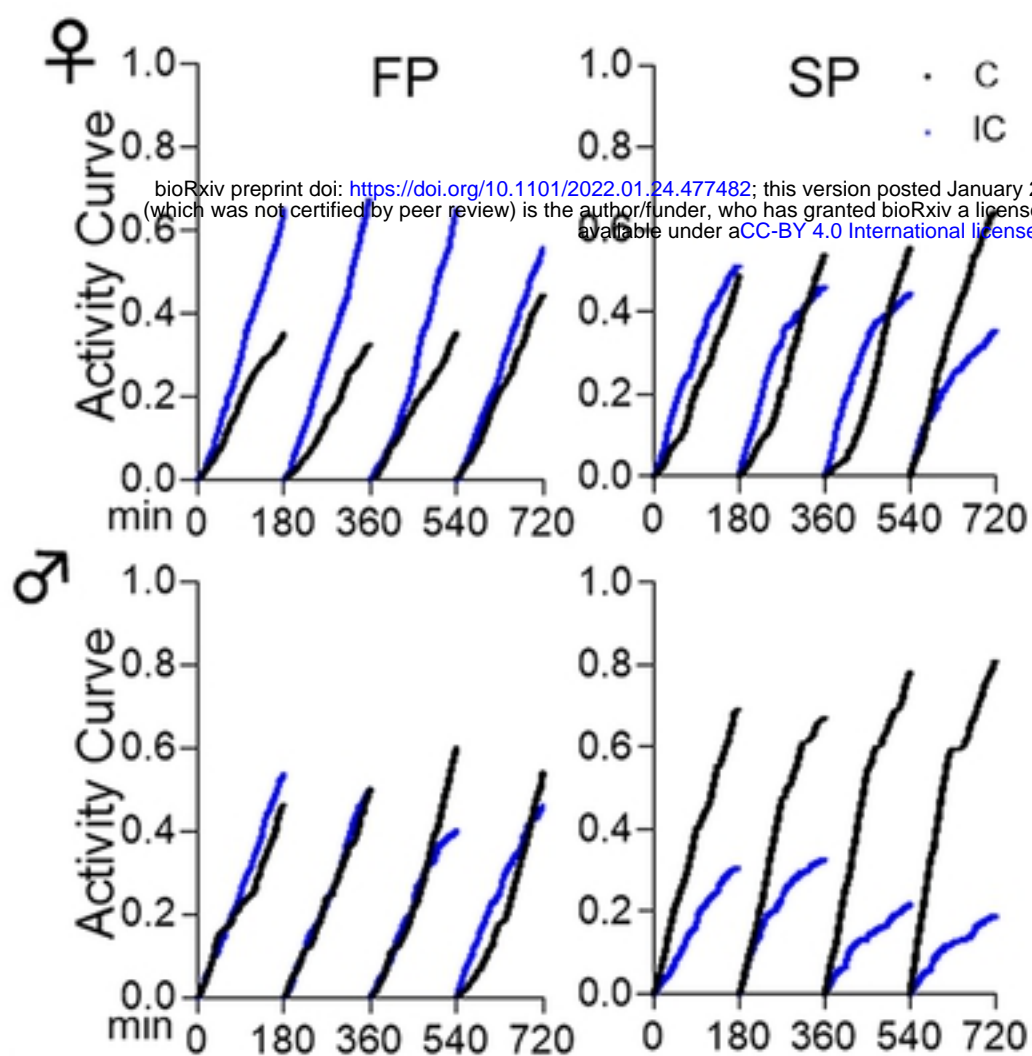
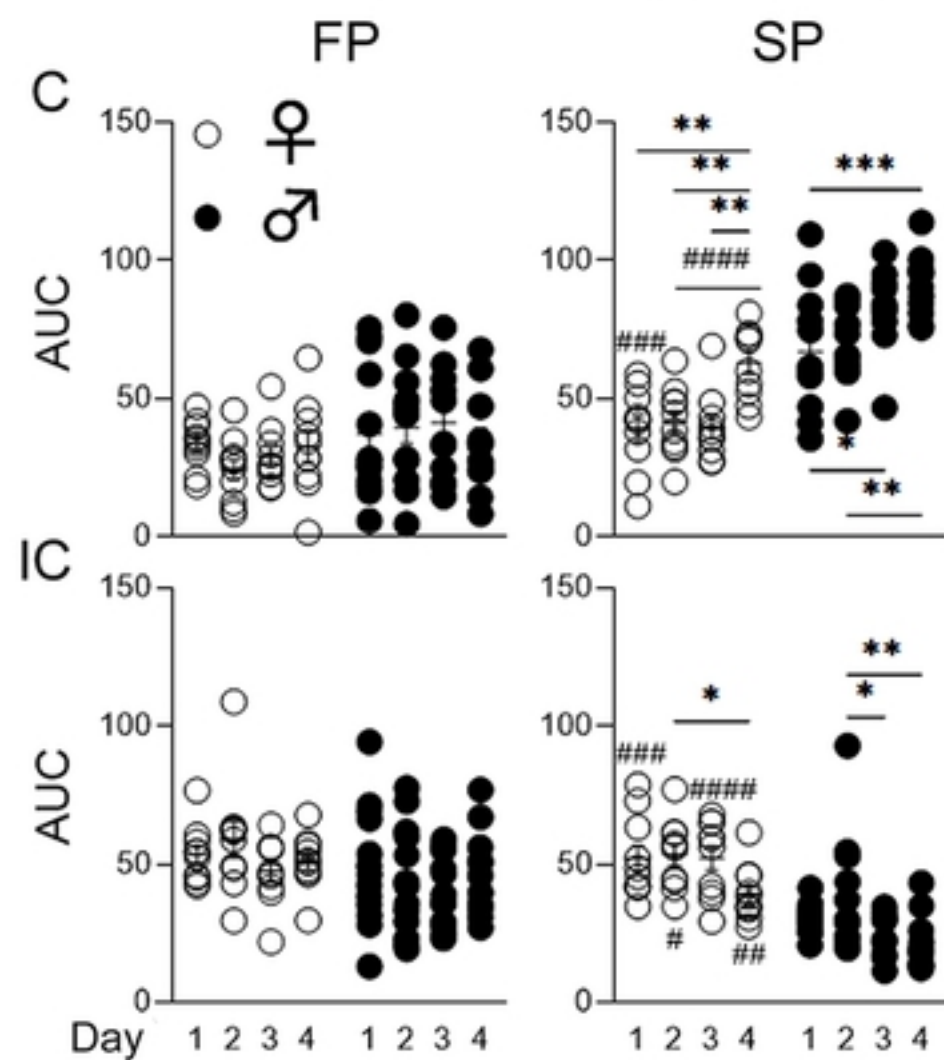


Figure 4

A



B



C

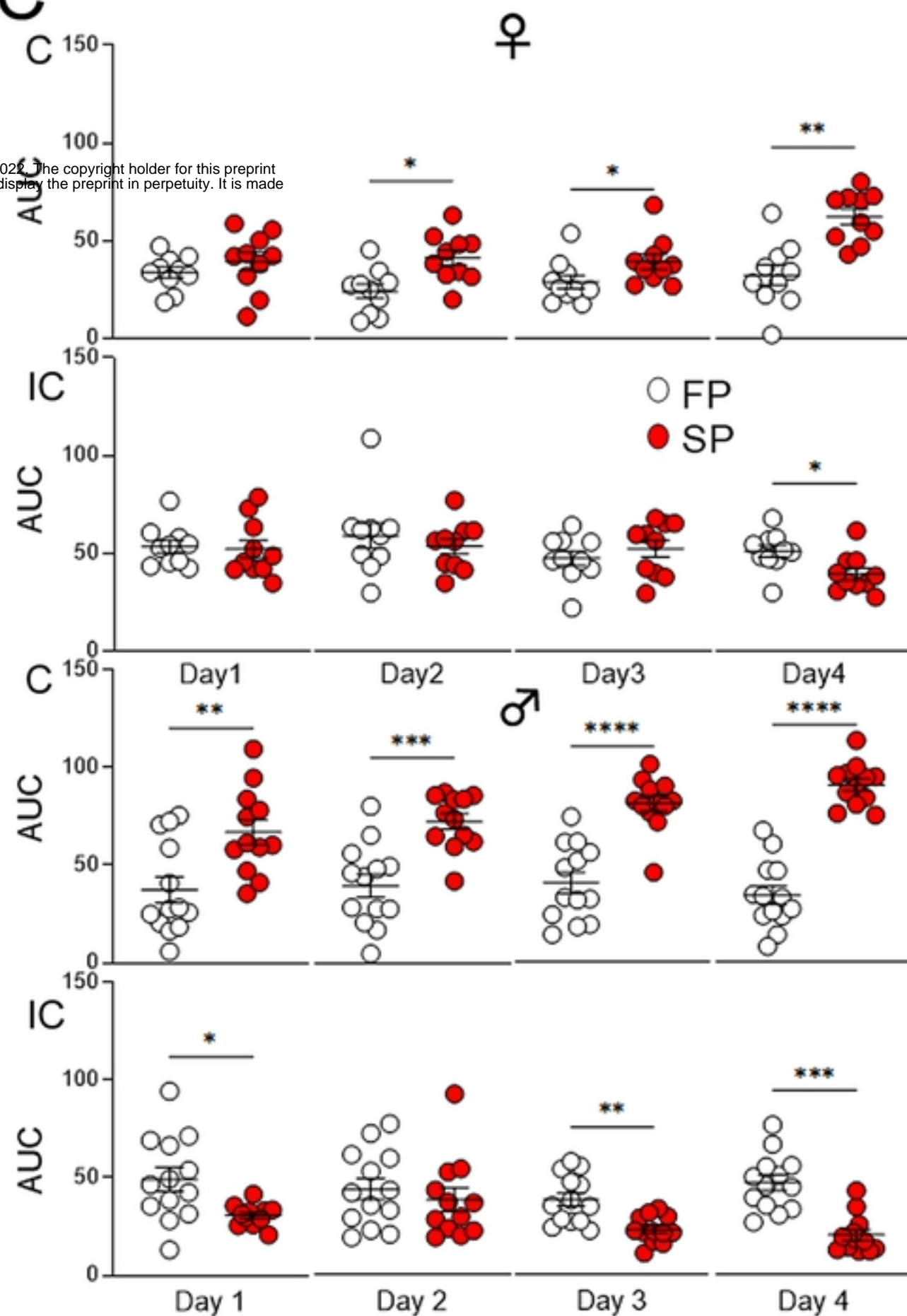


Figure 6

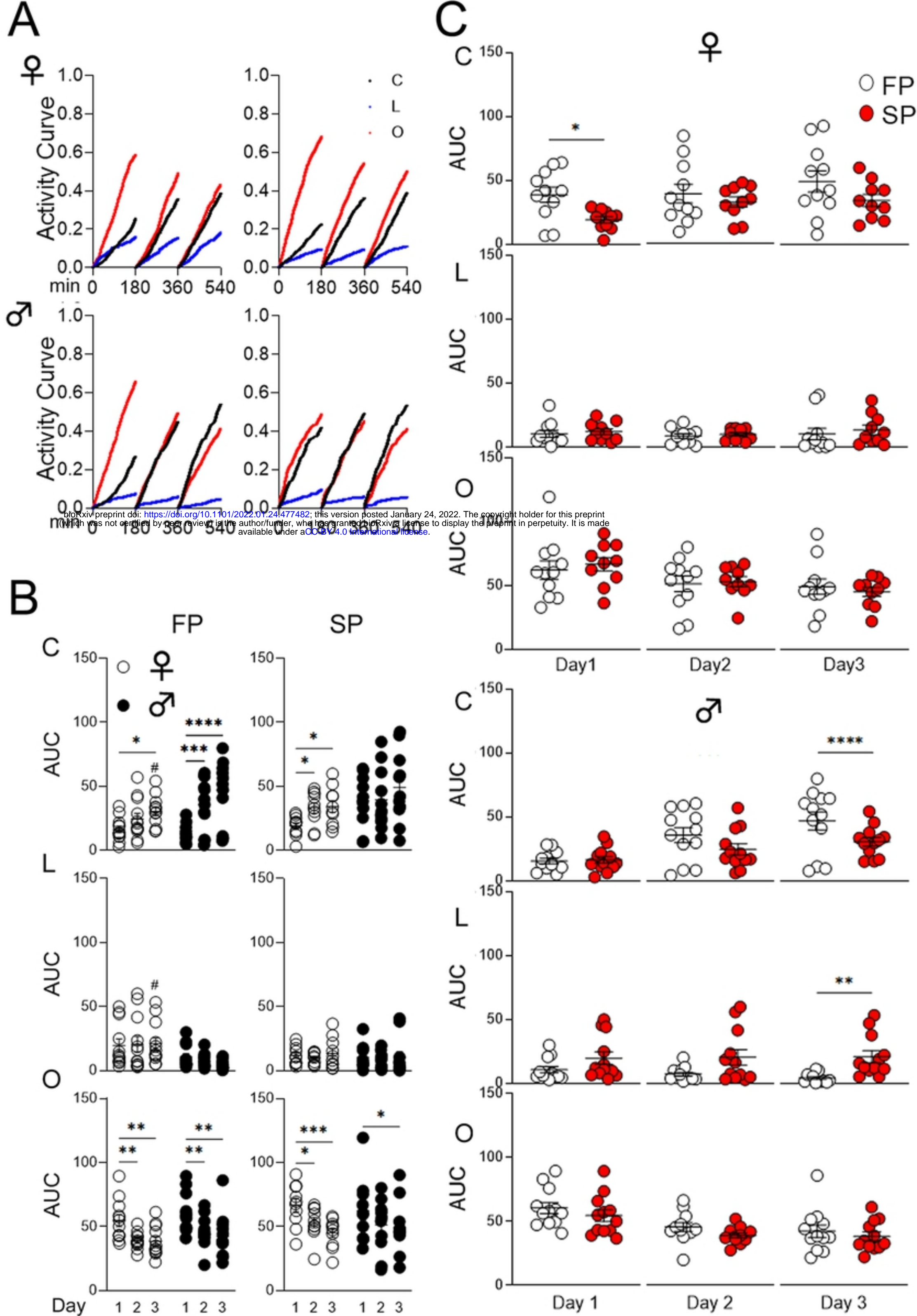


Figure 7



Figure 8

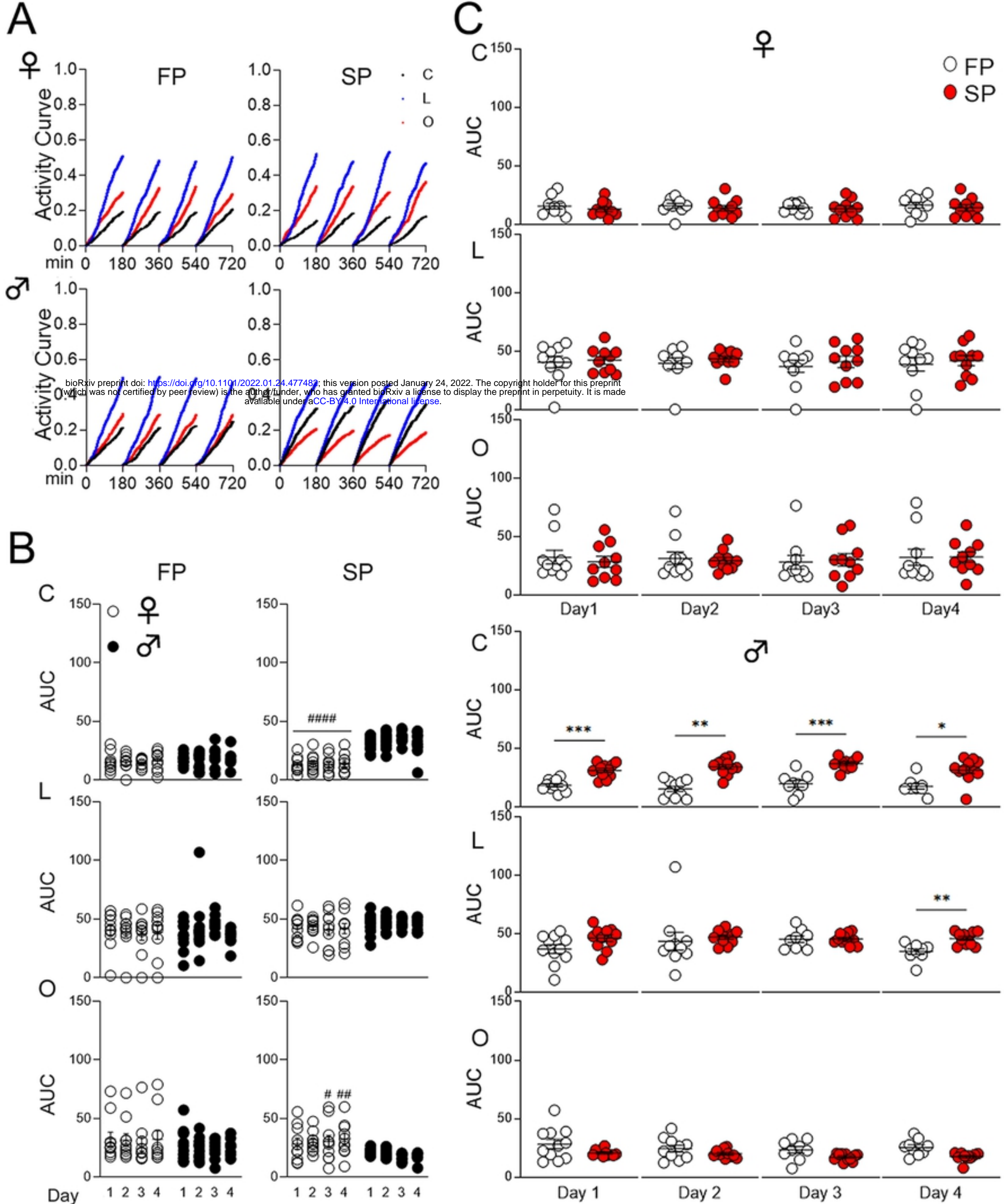


Figure 10

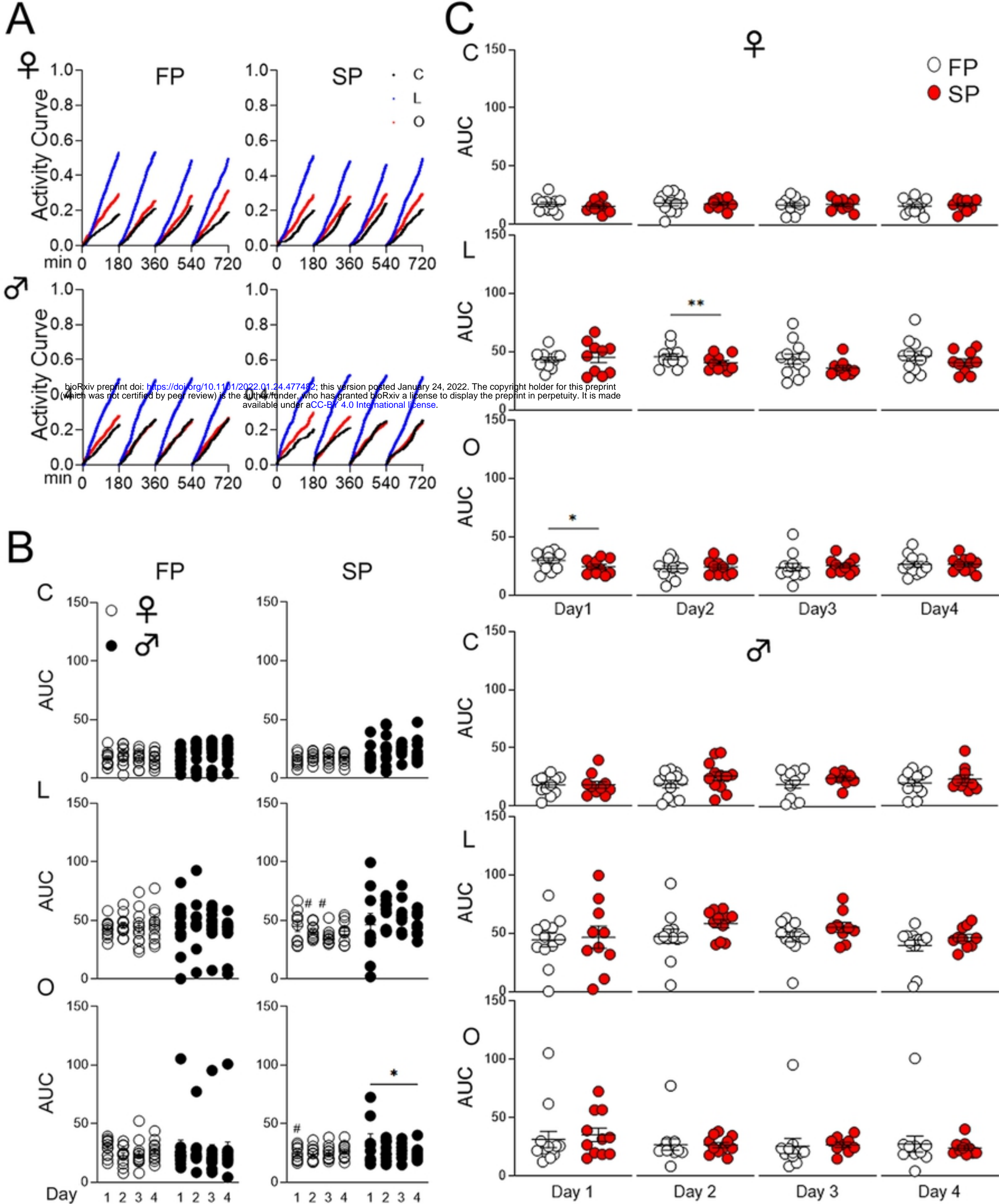


Figure 9

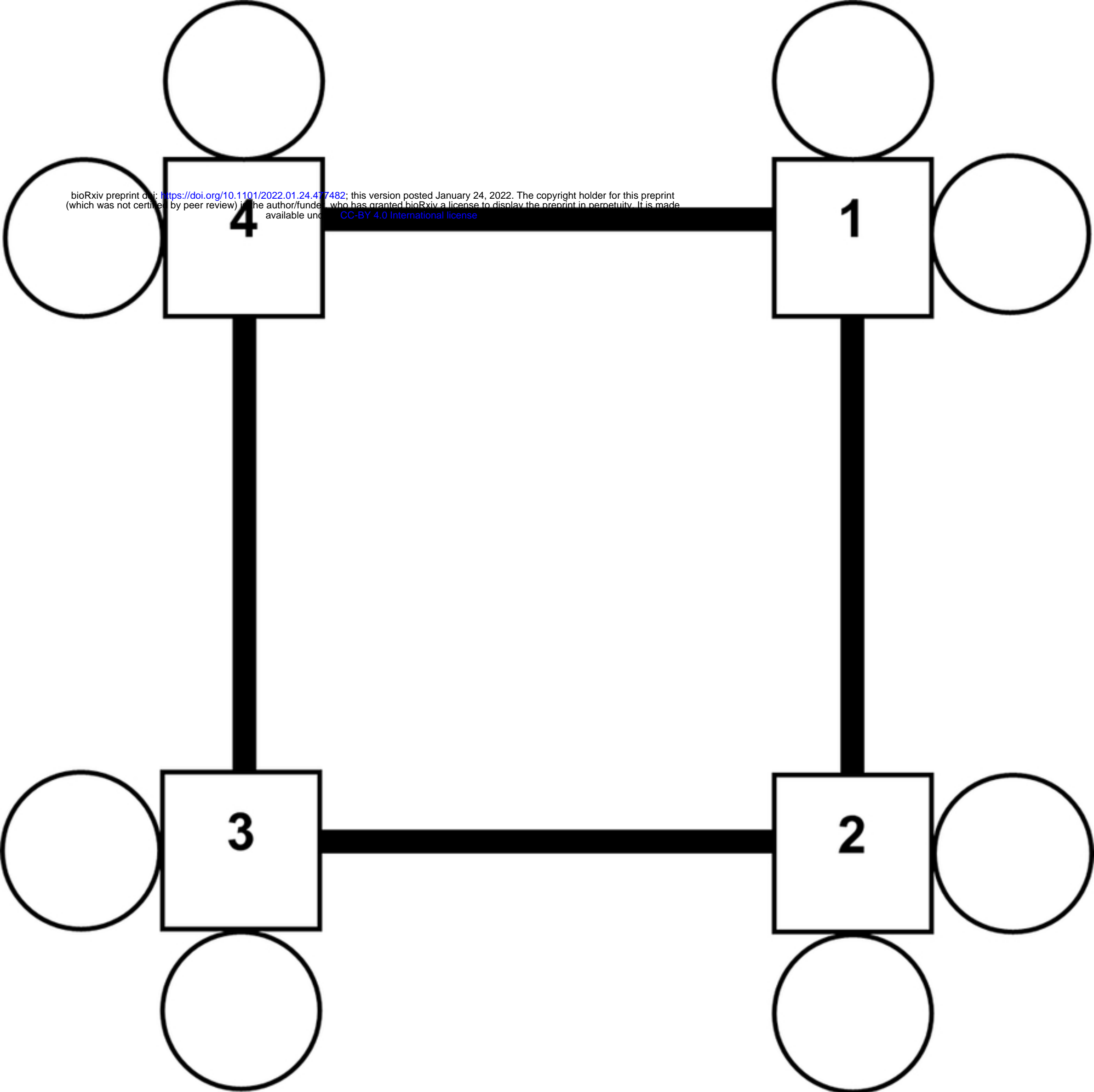


Figure 1

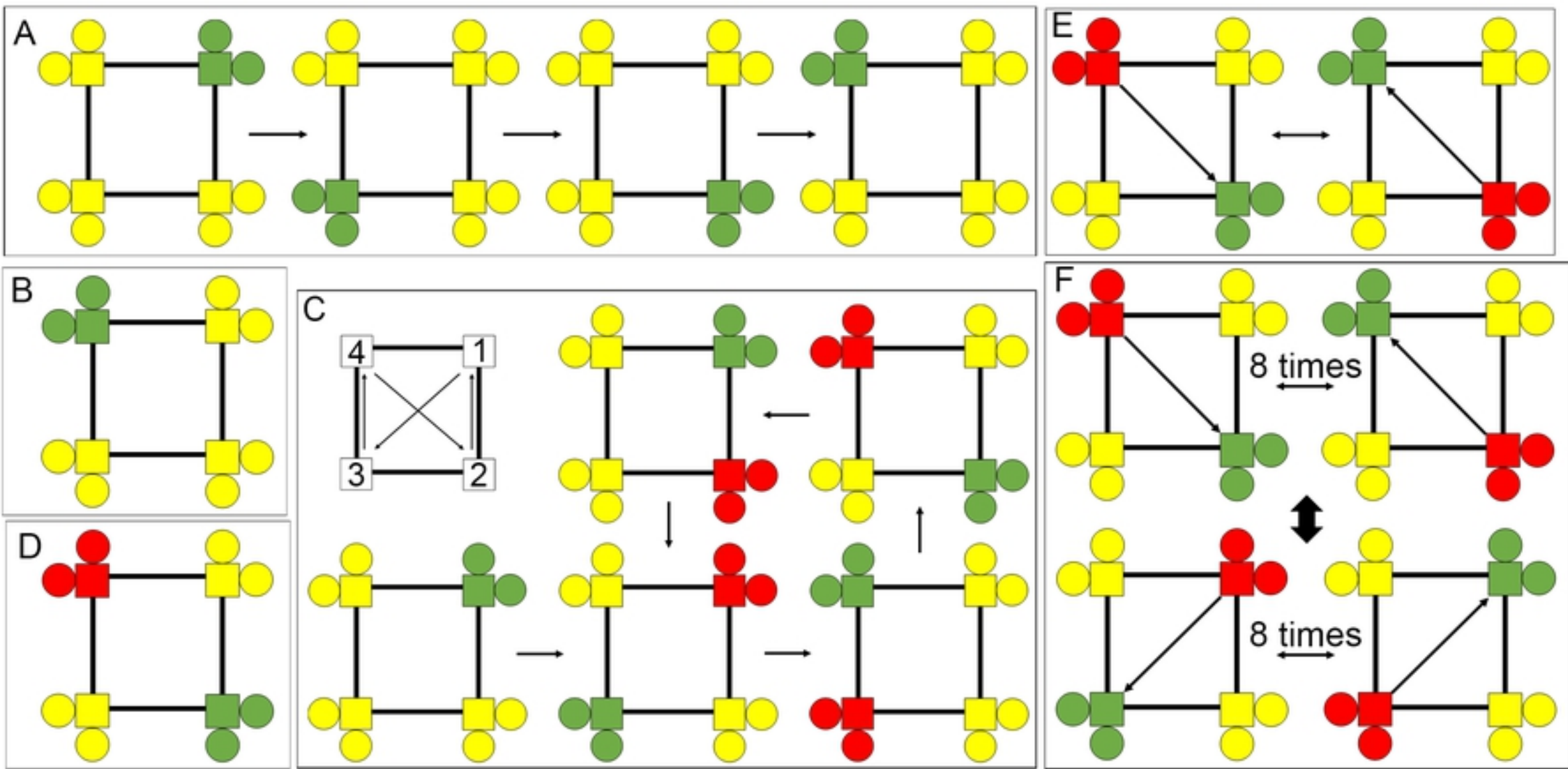


Figure 2

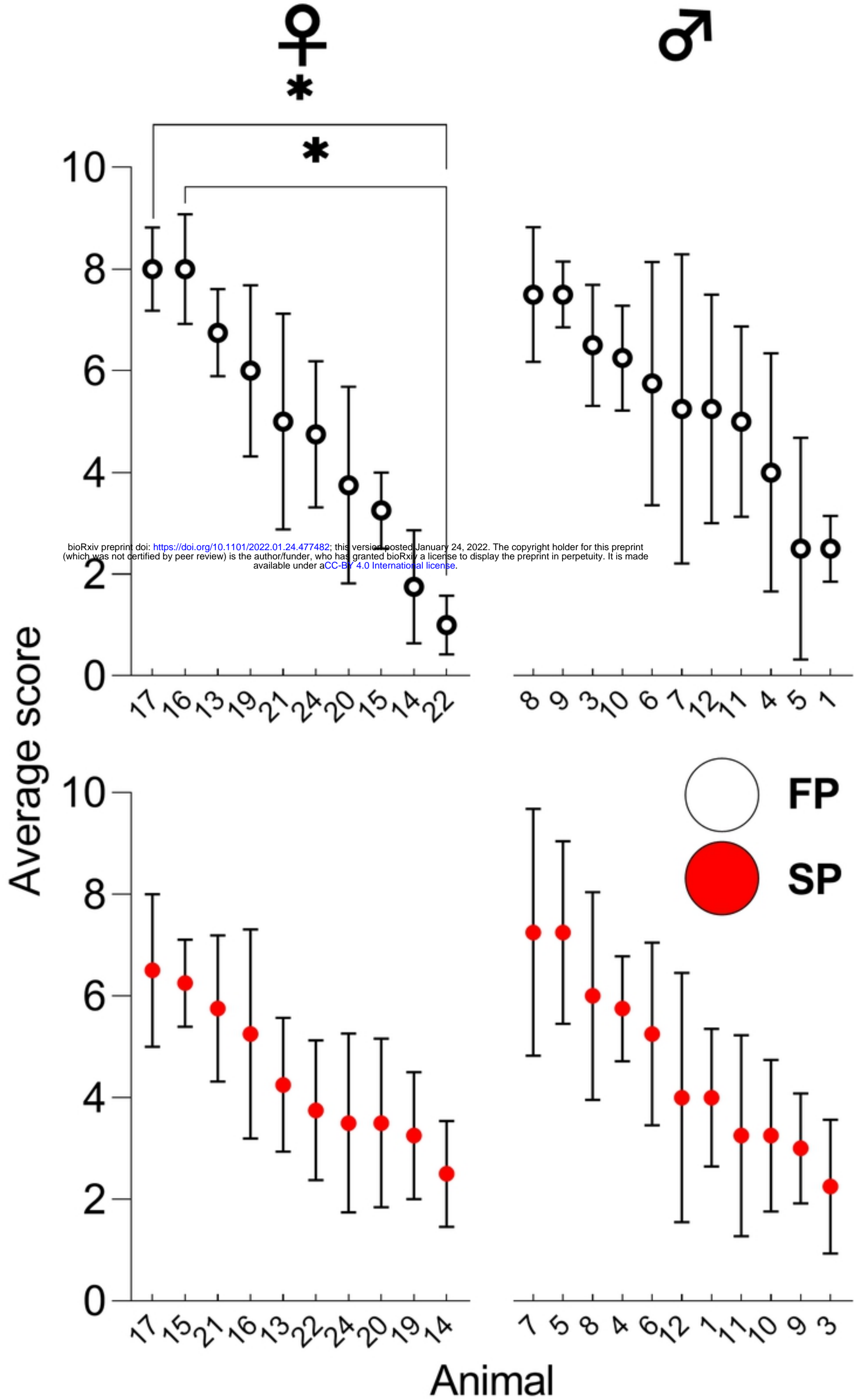


Figure 3