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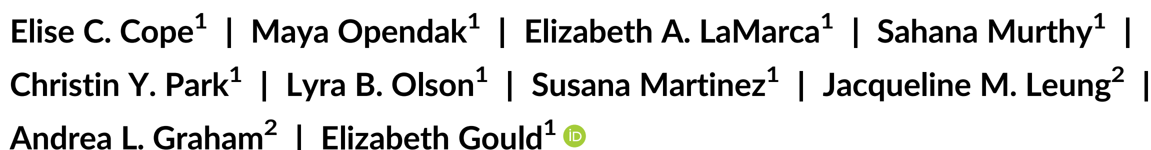
RESEARCH ARTICLE

The effects of living in an outdoor enclosure on hippocampal plasticity and anxiety-like behavior in response to nematode infection



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Abstract

The hippocampus of rodents undergoes structural remodeling throughout adulthood, including the addition of new neurons. Adult neurogenesis is sensitive to environmental enrichment and stress. Microglia, the brain's resident immune cells, are involved in adult neurogenesis by engulfing dying new neurons. While previous studies using laboratory environmental enrichment have investigated alterations in brain structure and function, they do not provide an adequate reflection of living in the wild, in which stress and environmental instability are common. Here, we compared mice living in standard laboratory settings to mice living in outdoor enclosures to assess the complex interactions among environment, gut infection, and hippocampal plasticity. We infected mice with parasitic worms and studied their effects on adult neurogenesis, microglia, and functions associated with the hippocampus, including cognition and anxiety regulation. We found an increase in immature neuron numbers of mice living in outdoor enclosures regardless of infection. While outdoor living prevented increases in microglial reactivity induced by infection in both the dorsal and ventral hippocampus, outdoor mice with infection had fewer microglia and microglial processes in the ventral hippocampus. We observed no differences in cognitive performance on the hippocampus-dependent object location task between infected and uninfected mice living in either setting. However, we found that infection caused an increase in anxiety-like behavior in the open field test but only in outdoor mice. These findings suggest that living conditions, as well as gut infection, interact to produce complex effects on brain structure and function.

KEYWORDS

adult neurogenesis, anxiety, hippocampus, microglia, parasite infection

1 | INTRODUCTION

The hippocampus of rodents exhibits considerable structural plasticity in adulthood. Neurons in the hippocampus undergo dendritic remodeling, synapse formation and elimination, as well as a substantial amount of neurogenesis throughout adult life (Gonçalves, Schafer, & Gage, 2016; Snyder & Cameron, 2012). New granule neurons are added to the dentate gyrus (DG) of the hippocampus in adulthood and these adult-generated neurons have been linked to several important functions of the hippocampus, including learning and memory (Deng, Aimone, & Gage, 2010; Denny, Burghardt, Schachter, Hen, & Drew, 2012; Snyder, Hong, McDonald, & Wojtowicz, 2005), as well as anxiety and stress regulation (Hill, Sahay, & Hen, 2015; Opendak & Gould, 2015; Schoenfeld & Gould, 2012; Snyder, Soumier, Brewer, Pickel, & Cameron, 2011; Surget et al., 2011). In addition to ongoing changes in the structure of neurons, the hippocampus exhibits a considerable amount of glial plasticity, in particular among microglia. Microglia, the brain's resident immune cells, constantly sense the environment and respond to environmental challenges with alterations in size, number, and shape (Nimmerjahn, Kirchhoff, & Helmchen, 2005;

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Norden, Muccigrosso, & Godbout, 2015; Tynan et al., 2010). In the hippocampus, microglia serve to clean up neuronal debris after damage occurs (Neumann, Kotter, & Franklin, 2009) and participate in adult neurogenesis by eliminating new neurons once they have died (Sierra et al., 2010). Changes in microglia morphology have also been linked to the functions of the hippocampus (Kohman, Bhattacharya, Kilby, Bucko, & Rhodes, 2013; Kreisel et al., 2014; McKim et al., 2016). Numerous studies have shown that both adult neurogenesis and microglial morphology are sensitive to a variety of experiences (Cameron & Schoenfeld, 2018; Kohman & Rhodes, 2013; Liu & Nusslock, 2018; Niraula, Sheridan, & Godbout, 2017), suggesting that these cells may play a role in experience-dependent alterations in hippocampal function.

A large literature suggests that living in a laboratory enriched environment has growth-promoting effects on the hippocampus compared to living in a standard laboratory cage (Kondo, 2017; Nithianantharajah & Hannan, 2006; Trinchero et al., 2017; Wu et al., 2008). While laboratory enriched environment paradigms vary widely, they typically include inanimate objects, social housing, and/or larger living spaces that allow for rodents to engage in more physical activity. Taken together, these studies have shown that enriched environment living alters multiple aspects of hippocampal structure, including increasing the complexity of dendrites and stimulating synaptogenesis (Birch, McGarry, & Kelly, 2013; Kondo, Takei, & Hirokawa, 2012; Leggio et al., 2005). Studies have also shown that enriched environment living enhances adult neurogenesis in the hippocampus (Drew et al., 2016; Kempermann, Kuhn, & Gage, 1997; Tashiro, Makino, & Gage, 2007), as well as diminishes reactivity of microglia (Chabry et al., 2015; Kohman, Bhattacharya, Wojcik, & Rhodes, 2013; Williamson, Chao, & Bilbo, 2012). These changes observed in hippocampal structure in response to increased environmental complexity are paralleled by alterations in behaviors associated with the hippocampus, including improved performance on certain cognitive tasks (Garthe, Roeder, & Kempermann, 2016; Kempermann, Gast, & Gage, 2002; Marlatt, Potter, Lucassen, & van Praag, 2012), as well as reduced anxiety-like behavior (Benaroya-Milshtein et al., 2004; Bhagya, Srikumar, Veena, & Shankaranarayana Rao, 2017; Rogers, Li, Lanfumey, Hannan, & Renoir, 2017). Collectively, these findings raise the possibility that enriched environment-induced alterations in both neurons and glia underlie behavioral changes linked to the hippocampus.

Studies of enriched environment living have attempted to identify individual factors underlying changes in brain structure and function, such as whether enhanced cognitive stimulation or increased physical activity are largely responsible for the observed brain effects. These studies have produced mixed results (Kobilo et al., 2011; Lambert, Fernandez, & Frick, 2005; Mustroph et al., 2012) and have raised the question of whether laboratory enriched environments accurately reflect experiences relevant to natural habitats. For instance, one aspect of natural living that is often intentionally minimized in laboratory enriched environments is that of chronic stress, a condition known to alter adult neurogenesis and microglial measures (reviewed in Opendak & Gould, 2015). While these studies have provided valuable information, they present only one side of the story and do not allow for an assessment of how living outdoors, where the environment is unstable and stress is common, alters brain structure and function.

Additional studies have shown that peripheral infection and overall illness are stressors that affect the brain and behavior (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; Sankowski, Mader, & Valdés-Ferrer, 2015). Experimentally induced sickness, through the injection of bacterial toxins, has been shown to diminish structural plasticity, including adult neurogenesis in the hippocampus (Bastos, Moriya, Inui, Katura, & Nakahata, 2008; Fujioka & Akema, 2010), as well as to impair cognitive function (Lee et al., 2008; Valero, Mastrella, Neiva, Sánchez, & Malva, 2014) and increase anxiety-like behavior (Bassi et al., 2012; Savignac et al., 2016). Enriched environment living has been shown to have impacts on the immune system by enhancing its ability to fight infection (Brod et al., 2017) and tumors (Garofalo et al., 2015), as well as diminishing inflammatory effects in certain brain regions (Mlynarik, Johansson, & Jezova, 2004; Williamson et al., 2012). Taken together, these findings suggest complex relationships among enrichment, stress, infection, and hippocampal structure and function, yet questions remain.

To address questions related to the effects of living in less controlled, more naturalistic environments on the brain and behavior, previous researchers have built outdoor enclosures in which to house experimental rodents (Spoelstra, Wikelski, Daan, Loudon, & Hau, 2016; Vyssotski et al., 2002). Findings from such studies are potentially meaningful because they better represent the complexity of living in the wild. In addition, since housing animals in a dynamic outdoor setting is likely to produce greater interanimal variability, detectable effects in such a setting would strengthen the validity of enriched environment effects detected in laboratory settings. To explore these relationships further, we used outdoor enclosures to mimic aspects of wild living compared to laboratory settings, with and without intestinal infection by nematodes and investigated the effects on adult neurogenesis, microglial plasticity, and behaviors associated with the hippocampus.

2 | MATERIALS AND METHODS

2.1 | Mice and nematode infection

All animal procedures were approved by the Princeton University IACUC and were in accordance with the guidelines of the National Research Council's Guide for the Care and Use of Laboratory Animals. The mice examined in this study are a subset of mice from the experimental design described in Leung et al. (2018). Adult, female C57BL/6 J mice (6–8 weeks old) were purchased from Jackson laboratories. To prevent the formation of a dominance hierarchy and excessive fighting in the outdoor enclosure, only female mice were used for these studies. For identification, all mice received ear tags and RFID transponders. To mimic local summer temperature and solstice daylight patterns during the experimental timeframe, mice were gradually acclimated and then kept at a room temperature of ~26 °C with a 15-hr light-9 hr dark cycle for 2 weeks before group assignment. Mice were randomly assigned to a housing group, either cage control or outdoors. A subset of mice from each housing group were infected by

oral gavage with the gut nematode Trichuris muris Strain E using a high dose of 200 embryonated eggs (Antignano, Mullaly, Burrows, & Zaph, 2011). Ten days after gut infection, mice in the outdoor group were moved from the laboratory cage setting to the outdoor enclosure, along with groups of uninfected mice (Figure 1). At the study endpoint (either ~3 weeks or ~4 weeks) postinfection, gut worm burdens were quantified as described previously (Leung et al., 2018). Mice in the cage control groups (uninfected and infected) remained group-housed (five per group) in the above laboratory conditions throughout the duration of the study. Indoor mouse cages were changed weekly.

2.2 | Outdoor animal enclosure

The outdoor enclosures consisted of replicate triangular-shaped pens arranged in a circle with natural soil and vegetation within each. Each triangle measured ~180 m^2 and was fenced by a 1.5 m-high zinced iron wall buried >80 cm deep and topped with electrical fencing to keep out terrestrial predators. Aluminum pie plates were strung across the top of the enclosure with fishing line to deter aerial predators. A small (180 × 140 × 70 cm) straw-filled shed was present in each enclosure, along with two watering stations and a feeding station, in which standard rodent chow was provided ad libitum (PicoLab Rodent Diet 20) (Figure 1). Antenna readers were placed around the food site to identify RFID-chipped mice. Mice living in the outdoor enclosure, however, also had access to food sources found within the pens, including berries, seeds, and insects. Each triangle housed mice in groups of 11–12 that were separated by infection status to ensure no parasitic worms were transmitted to the uninfected group. Six out of the eight available wedges were used for this study. For trapping mice, the food silo that typically provided chow for the mice was removed on trapping nights. Longworth traps baited with standard rodent chow were used to catch mice weekly for weighing and sampling and again at the endpoint of the experiment; approximately two baited traps were set per mouse per enclosure in the early evening, and all traps were checked within 12 hr.

2.3 | Behavioral testing

Approximately 10–19 days after transfer to the outdoor enclosure (or 20–29 days postinfection), mice were captured and transported to the laboratory in the morning (Figure 1). Subsets of mice in the outdoor pens were captured over the course of 9 days. Behavioral testing started that night (within 24 hr of capture), during the active cycle for mice. Mice were first tested for cognitive behavior using the hippocampus-dependent object location (OL) test (Barker & Warburton, 2011). Immediately after cognitive testing, mice were placed in an open field (OF) apparatus to investigate anxiety-like behavior. All testing arenas and objects were cleaned with 70% ethanol between mice. All behavior was recorded on video camera and scored by an investigator blind to experimental group condition. Cage control mice were tested in a similar manner, interleaved among the outdoor mice.

2.3.1 | OL testing

The OL testing apparatus was an open-field box (23 × 25 × 25 cm). Throughout habituation and testing, the room lighting remained low and mice were placed in the boxes in the same orientation. Objects that were <8 cm in height or width and had varying 3D surfaces for exploration were used for testing. To give the mice some familiarity to objects prior to testing, objects (different than those used during the test) were placed in the home cage several hours before behavioral testing began. Mice were also habituated to the testing arena by

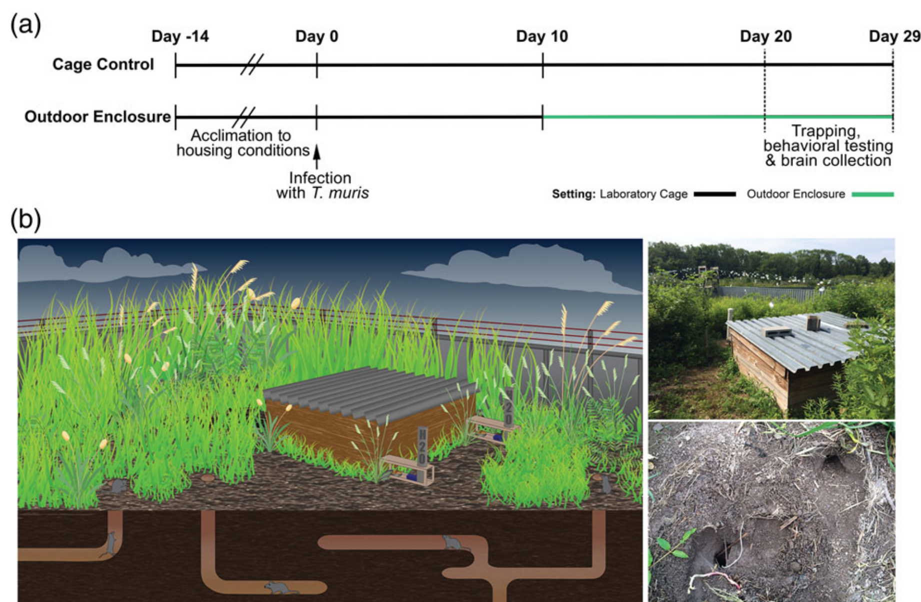

FIGURE 1 Experimental timeline and outdoor field apparatus. (a) Timeline for gut parasite infection, transfer to the outdoor enclosure, and behavioral testing. (b) Left, schematic of the outdoor living enclosure with the shed, watering/feeding stations, vegetation, and burrows. The burrows and mice are enlarged relative to the rest of the schematic of the enclosure. Top right, photograph of a triangle enclosure from the outdoor apparatus. Bottom right, picture of two burrows presumably made by mice in a triangle of the outdoor apparatus. Photograph credits: W. C. Craigens [Color figure can be viewed at wileyonlinelibrary.com]

placing them in the arena for 10 min three separate times. After habituation to the arena and objects, mice underwent testing, which consisted of a familiarization phase and a test phase. Objects were placed alongside one wall of the arena ~6 cm away from the corners and 10 cm from each other. During the familiarization phase, mice were exposed to two identical objects until their cumulative investigation time for both objects reached 30 s, or until a total of 10 min had elapsed. After the familiarization phase, mice were returned to their home cage for 5 min, and then placed back into the arena for a 2 min test. During the test phase, mice were exposed to the same objects used in the familiarization phase. One of the objects remained in the same location and orientation, while the other was rotated 180° and moved to the opposite wall such that the objects were diagonal to each other. The location of the novel orientation within the arena was counterbalanced for all tests. Cognitive performance was determined using a discrimination ratio (DR), which was calculated by dividing the difference in time exploring the novel and familiar locations by the total time exploring both locations. Object exploration was defined as directing the nose toward the object at less than or equal to 2 cm.

2.3.2 | OF test

Mice were tested for anxiety-like behavior in an OF apparatus as previously described (Kulesskaya & Voikar, 2014; Seibenhener & Wooten, 2015). Mice were individually placed in the center of a brightly lit, OF arena (43 × 43 × 43 cm) constructed out of Plexiglas and were allowed to explore for 10 min. Using video tracking software (Biobserve), the amount of time and the number of entries into the center portion of the arena was recorded for each mouse. Total distance traveled in the whole arena was used to measure locomotor activity.

2.4 | Immunohistochemistry

The morning following behavioral testing, mice were euthanized by CO_2, and the brains were extracted. All brains were postfixed in 4% paraformaldehyde for 72 hr followed by cryoprotection in 30% sucrose for 48 hr. Forty micrometer thick coronal sections were collected on half-brains throughout the rostrocaudal extent of the hippocampus using a cryostat (Leica). For immature neuron immunolabeling, free-floating sections were rinsed in Tris-buffered saline (TBS) and then incubated with .5% Tween-20, 3% normal donkey serum, and goat anti-doublecortin (DCX; 1:100; Santa Cruz Biotechnology) in TBS at 4 °C for 48 hr. Sections were then washed and incubated with Alexa Fluor donkey anti-goat 568 (1:250; Invitrogen) for 1 hr at room temperature. Immunolabeling for microglia was conducted separately on free-floating sections that were rinsed in PBS, incubated with 3% normal donkey serum, PBS with .1% Triton X-100, and rabbit anti-ionized calcium-binding adapter molecule 1 (iba1; 1:500; Wako), a microglial cell body marker, and rat anti-CD68 (1:200; Serotec), a microglial lysosomal marker used to assess microglial reactivity, for 24 hr at 4 °C. Tissue was then rinsed and incubated with secondary antisera, consisting of Alexa Fluor donkey anti-rabbit 488 (1:250; Invitrogen) and Alexa Fluor donkey anti-rat 568 (1:250; Invitrogen). After immunolabeling, sections were washed, mounted onto Suprafrost Plus slides, dried, and counterstained with Hoechst 33342 (Molecular Probes) 1:1,000 in water. Slides were then coverslipped over glycerol in PBS (3:1).

2.5 | Cell counting

Since the dorsal hippocampus has been implicated in cognitive function and the ventral hippocampus has been implicated in anxiety and stress regulation (Fanselow & Dong, 2010), all histological measures in these regions were analyzed separately. Cell densities for immature neurons (DCX) and microglia (iba1) were determined throughout the entire rostrocaudal extent of the DG (Franklin & Paxinos, 2008) on every sixth section using a BX-60 Olympus microscope assisted by Stereo Investigator software (Microbrightfield Bioscience). The reference space (contour) for the granule cell layer was delineated by outlining at low power (4× objective). Counts of iba1-positive cells were obtained using a 40× objective and counts of DCX-positive cells were obtained using a 100× oil objective. Cell densities were determined for each mouse by taking the total number of positively labeled cells and dividing it by the volume of the granule cell layer (contour area of the counted sections multiplied by 40 for thickness of cut section). To determine the total volume of the granule cell layer, the summed contour areas were multiplied by 480 (40 for thickness of cut section, 6 for interval between sections, and 2 for bilateral).

2.6 | Microglial morphology and reactivity analyses

Microglia were analyzed for the number of primary processes, cell body area, and activation status from tissue double-labeled with iba1 and CD68. For each animal and brain region, 10 randomly selected iba1-positive microglial cells were obtained from z-stack images with a Zeiss confocal microscope (LSM 700) using a 63× oil objective with a 1.2× zoom factor and a .56 μm *z*-step. Cells selected for imaging were located toward the middle portion of the z-plane to fully analyze the microglia processes. Cross-sectional cell body area was measured using the polygon selection tool in ImageJ (NIH). Iba1-positive microglial cells colabeled with the lysosomal marker CD68, a marker of microglial activation, were assessed from maximum intensity *z*-projections of the image stacks created using ImageJ (NIH). CD68 aggregates (defined as staining that was >3 μm^2 in area) were manually counted from the cell body and the processes of each individual microglial cell (Cope et al., 2016).

2.7 | Statistical analysis

The investigators were blind to the experimental group throughout data collection and analysis. All data sets were examined for normal distribution and homogeneity of variance using Levene's tests. Any data that did not meet the assumptions for parametric tests were log transformed prior to statistical analyses. All histological data were analyzed using two-way ANOVAs followed by Tukey post-hoc comparisons. Because mice that lived outside, but not those that lived in the laboratory control cages, were subjected to the stress of capture and transport prior to behavioral testing, comparisons between uninfected and infected mice were done separately for cage control and outdoor enclosure mice. Two outliers were identified in the OF test

and removed from any of the OF behavioral analyses using a Rout outlier test with Q = 1%. Behavioral data sets were analyzed using unpaired Student's t-tests with the exception of one dataset that did not meet parametric assumptions and were not appropriate for log transformation. Those data were analyzed using a Mann–Whitney U test. Pearson's rank correlation coefficient test was used to analyze the association between OF anxiety-like behavior and OL scores, as well as between anxiety-like behavior and changes in microglia in the ventral hippocampus. Significance was defined as $p < .05$. Graphs were prepared using Graph Pad software. All statistics were completed using either R studio 3.0.1 or Graph Pad Prism 6.0 (Graph Pad Software, Inc). N sizes, p-values, and statistical tests for each experiment are reported either in the figure legend or in the Results section.

3 | RESULTS

3.1 | Outdoor mice had comparable body weights but higher worm burdens at the end of the study compared to their cage control counterparts

We observed evidence that mice living in the outdoor enclosure engaged in typical behaviors of mice living in the wild such as burrowing (Figure 1b). Furthermore, all captured mice living in the outdoor enclosure appeared to be healthy in terms of the quality of their fur and the lack of overt wounds or signs of dermatologic problems. While initially there was a decrease in body weight after mice were moved outdoors (body weight - cage control uninfected: 19.84 ± .52 g, outdoor enclosure uninfected: 17.95 ± .31 g, cage control infected: 19.88 ± .27 g, outdoor enclosure infected: 17.92 ± .29 g; effect of setting: $F_{(1,38)}$ = 31.74, $p < .0001$, effect of infection: $F_{(1,38)}$ = .0003, p = .99, interaction of setting vs. infection: $F_{(1,38)}$ = .009, p = .92; Leung et al., 2018), by the end of the study period, outdoor mice had comparable body weights to those living in laboratory cages (body weight - cage control uninfected: 19.88 ± .33 g, outdoor enclosure uninfected: 19.15 ± .53 g, cage control infected: 20.04 ± .32 g, outdoor enclosure infected: 18.85 ± .45 g; effect of setting: $F_{(1,38)}$ = 4.04, p = .051, effect of infection: $F_{(1,38)}$ = .022, p = .88, interaction of setting vs. infection: $F_{(1,38)}$ = .24, p = .63; Leung et al., 2018). Infected mice living in both settings exhibited evidence of intestinal nematodes at the end of the study period, although those living in the outdoor enclosure had a significantly higher worm burden than those living in cages (average number of worms - cage control: 1.93 ± 1.14, outdoor enclosure: 26.35 ± 9.33; $t_{[30]}$ = 2.44, p = .021; Leung et al., 2018).

3.2 | Living in an outdoor enclosure increased the numbers of doublecortin-positive neurons in the dorsal and ventral hippocampus compared to living in cage control conditions

Analysis of the density of DCX+ neurons in the dorsal DG revealed a significant overall difference between mice living in standard cage control conditions and those living in an outdoor enclosure ($F_{[1,25]}$ = 13.61, p = .0011) (Figure 2a,b). In both uninfected and infected mice, living outside produced an overall increase in the density of juvenile neurons compared to living in a cage. No significant main effects of infection ($F_{[1,25]}$ = .2962, p = .5911) or significant interaction effects (setting vs. infection) ($F_{[1,25]}$ = .1730, p = .6810) were observed (Figure 2a,b). No differences were observed in the volume of the dorsal granule cell layer suggesting that increases observed in DCX+ cell density in outdoor mice reflect increases in the number of immature neurons (cage control uninfected: .65 ± .025 mm^3, cage control infected: .59 ± .016 mm^3,

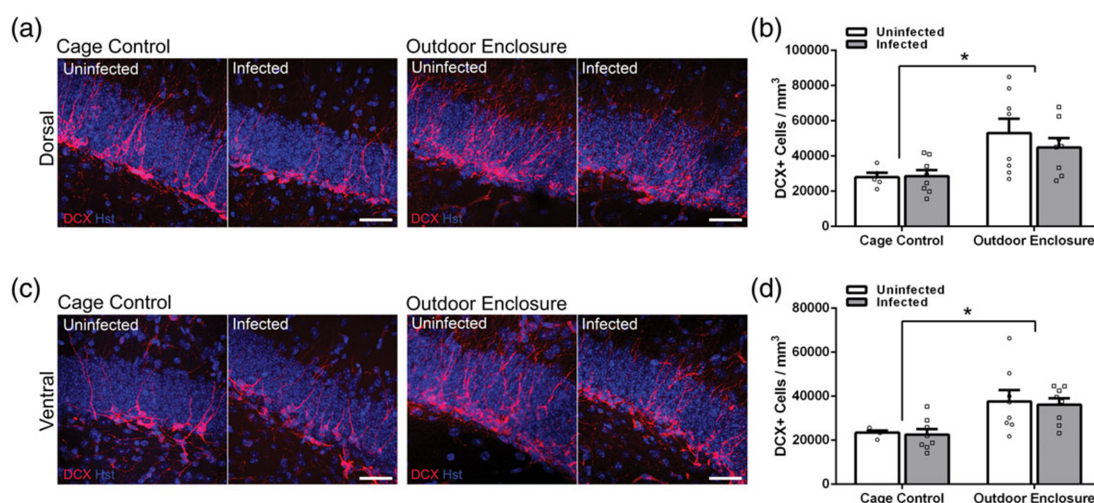

FIGURE 2 Mice living in an outdoor enclosure had increased numbers of juvenile neurons in the hippocampus. (a) Confocal images of brain sections from uninfected and infected mice living in either cage control or the outdoor enclosure conditions immunolabeled with the immature neuron marker DCX (red) and counterstained with Hoechst 33342 (blue) in the dorsal DG. (b) Mice living in the outdoor enclosure had increased numbers of DCX+ cells in the dorsal DG. *p = .0011. (c) Confocal images of brain sections from uninfected and infected mice living in either cage control or the outdoor enclosure conditions immunolabeled with the DCX and counterstained with Hoechst 33342 in the ventral DG. (d) Mice living in the outdoor enclosure had increased numbers of DCX+ cells in the ventral DG. *p = .0003. Scale bar equals 40 μm. For all panels, n = 5 for uninfected cage control and n = 8 for all other groups (infected cage control, uninfected outdoor enclosure, infected outdoor enclosure). Bars represent mean + SEM [Color figure can be viewed at wileyonlinelibrary.com]

outdoor uninfected: .63 ± .028 mm^3, outdoor infected: .60 ± .027 mm^3; $F_{[1, 22]}$ = .1955, p = .6627). Similar effects were observed in the ventral hippocampus; living in the outdoor enclosure increased the number of immature neurons compared to living in control cages ($F_{[1,25]}$ = 17.38, p = .0003) with no main effects of infection status ($F_{[1,25]}$ = .1606, p = .6920) or interaction (setting vs. infection) ($F_{[1,25]}$ = .1135, p = .7390) (Figure 2c,d). Again, no differences were observed for the ventral granule cell layer volume suggesting that increases in DCX+ cell density reflect overall increases in immature neuron number (cage control uninfected: .40 ± .030 mm^3, cage control infected: .35 ± .015 mm^3; outdoor uninfected: .34 ± .04 mm^3, outdoor infected: .36 ± .026 mm^3; $F_{[1, 23]}$ = .8231, p = .3737).

3.3 | Living in the outdoor enclosure diminishes infection-induced increases in microglial reactivity

Analysis of CD68 aggregate labeling within iba1+ microglia of the dorsal hippocampus showed that mice living in the outdoor enclosure had overall lower microglial reactivity compared to those living in the laboratory cage ($F_{[1,23]}$ = 12.95, p = .0015) (Figure 3a,b). No significant main effects were noted for infection status ($F_{[1,23]}$ = 3.350, p = .0802) or interaction (setting vs. infection) ($F_{[1,23]}$ = 2.091, p = .1617) (Figure 3a,b). By contrast, in the ventral hippocampus, no significant main effect of setting was observed ($F_{[1,23]}$ = .3342, p = .5688), whereas a main effect of infection was noted ($F_{[1,23]}$ = 12.20, p = .0020) with a significant interaction (setting vs. infection) as well ($F_{[1,23]}$ = 4.374, p = .0477) (Figure 3c,d). A significant increase in microglial reactivity was observed between infected mice and uninfected mice living in cages (p = .0043) but not between infected and uninfected mice living outside (p = .7404) (Figure 3c,d).

3.4 | Gut nematode infection reduced the number of microglia and microglial processes in the ventral hippocampus of mice living in an outdoor enclosure

Analysis of the density of microglia revealed surprising reductions in both the dorsal and ventral hippocampus of infected compared to uninfected mice living in the outdoor enclosure. Significant main effects of infection were observed for dorsal ($F_{[1,23]}$ = 18.44, p = .0003) (Figure 4a) and ventral ($F_{[1,23]}$ = 17.11, p = .0004) (Figure 4d) DG. No significant main effects of setting were observed for dorsal ($F_{[1,23]}$ = 1.066, p = .3126) and no interaction was observed ($F_{[1,23]}$ = 1.227, p = .2794) (Figure 4a). By contrast, for the ventral DG, there were significant main effects of setting ($F_{[1,23]}$ = 8.186, p = .0088) but again, no interaction effects ($F_{[1,23]}$ = 1.793, p = .1936) (Figure 4d).

Analysis of primary processes on microglial cells revealed no significant main effects of setting ($F_{[1,23]}$ = .5710, p = .4575), infection ($F_{[1,23]}$ = .08130, p = .7781), or an interaction between setting and infection ($F_{[1,23]}$ = .008262, p = .9284) for dorsal DG (Figure 4b). However, a significant main effect of infection was observed for ventral DG ($F_{[1,23]}$ = 6.957, p = .0147) with no main effect of setting ($F_{[1,23]}$ = .08125, p = .7782) or interaction between the two factors (infection vs. setting: $F_{[1,23]}$ = 1.821, p = .1903) (Figure 4e). No significant main effects of infection or setting were observed for microglial cell body area in either dorsal (infection: $F_{(1,23)}$ = .0001812, p = .9894; setting: $F_{(1,23)}$ = .1507, p = .7015; setting vs. infection: $F_{(1,23)}$ = .2635, p = .6127) (Figure 4c) or ventral (infection: $F_{(1,23)}$ = .3517, p = .5589); setting:

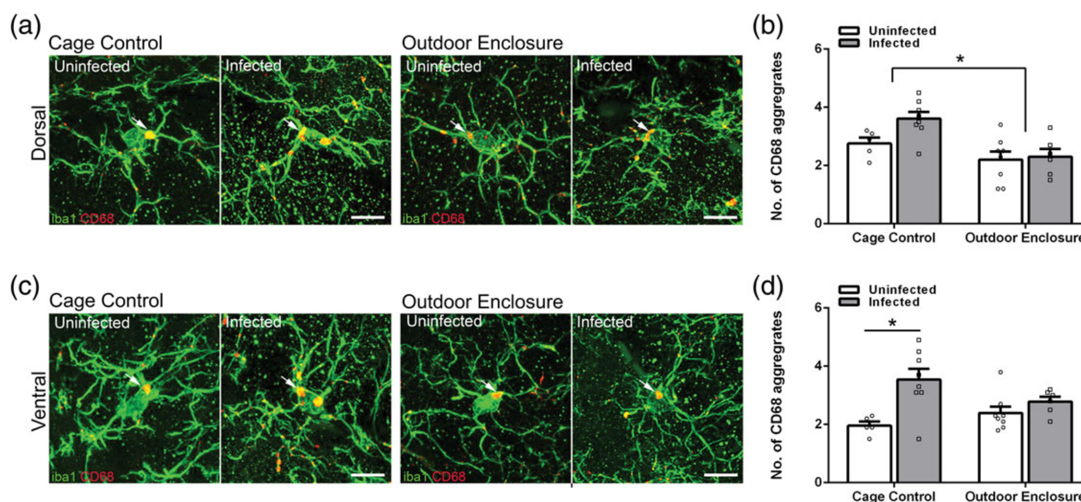

FIGURE 3 Mice living in the outdoor enclosure had reduced microglial reactivity in the hippocampus despite parasitic worm infection. (a) Confocal images of brain sections from uninfected mice and infected mice living in either cage control or the outdoor enclosure conditions immunolabeled with microglia marker iba1 (green) and the microglial lysosomal marker CD68 (red) in the dorsal DG. (b) Mice living in the outdoor enclosure had fewer CD68 aggregates in response to parasite infection in the dorsal DG. *p = .0015. (c) Confocal images of brain sections from uninfected mice and infected mice living in either cage control or the outdoor enclosure conditions immunolabeled with iba1 and CD68 in the ventral DG. (d) Mice living as cage controls with parasite infection had more CD68 aggregates in the ventral DG, an effect which was prevented by living in the outdoor enclosure. *p = .0043. Scale bar equals 10 μm. For all panels, n = 5 for uninfected cage control, n = 8 infected cage control and uninfected outdoor enclosure, n = 6 for infected outdoor enclosure. Bars represent mean + SEM [Color figure can be viewed at wileyonlinelibrary.com]

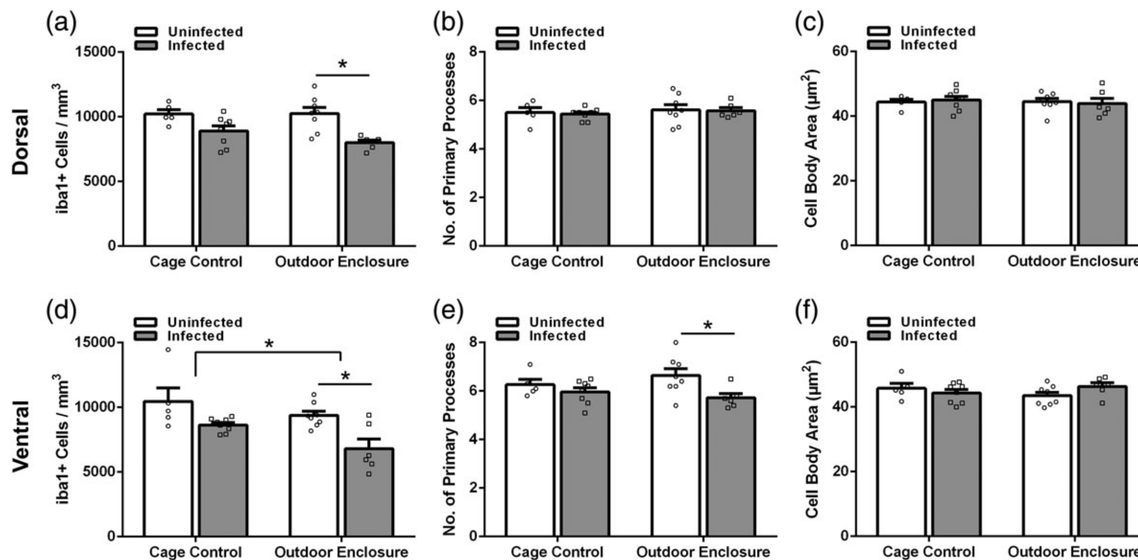

FIGURE 4 Mice with gut nematode infections living in an outdoor enclosure had reduced numbers of microglia. (a) Mice living outside had fewer numbers of iba1+ microglia in the dorsal DG in response to parasite infection.*p = .0035. (b) The number of primary processes of iba1+ microglia in the dorsal DG was not changed by living setting or parasite infection. (c) The cell body area of iba1+ microglia in the dorsal DG was not changed by living setting or parasite infection. (d) Mice living in the outdoor enclosure had fewer iba1+ microglia in the ventral DG in response to parasite infection. *p = .0088 for cage control versus outdoor enclosure and *p = .0031. (e) Mice living outside had fewer primary processes of iba1+ microglia in the ventral DG in response to parasite infection. *p = .0377. (f) The cell body area of iba1+ microglia in the ventral DG was not changed by living setting or parasite infection. For all panels, n = 5 for uninfected cage control, n = 8 infected cage control and uninfected outdoor enclosure, n = 6 for infected outdoor enclosure. Bars represent mean + SEM

$F_{(1,23)}$ = .01460, p = .9049; setting vs. infection: $F_{(1,23)}$ = 3.148, p = .0892) (Figure 4f) subregions.

3.5 | Gut nematode infection increases anxiety-like behavior in mice living in an outdoor enclosure

Analysis of cage control housed mice performing the OL task revealed no significant differences between uninfected and infected groups in their ability to discriminate objects in familiar locations versus objects in novel locations (DR) ($t_{[16]}$ = .147, p = .885) or in time spent exploring objects ($t_{[16]}$ = 1.37, p = .189) (Figure 5a). Likewise, the DR was not significantly different between uninfected and infected mice living in the outdoor enclosure (U = 43, p = .1545), but it is worth noting that the infected mice living in the outdoor enclosure showed significantly higher variability in terms of their DR (Levene's test, $F_{[1,22]}$ = 7.79, p = .011) (Figure 5b). Furthermore, infected mice living in the outdoor enclosure explored the objects for significantly less time during the testing phase than uninfected mice ($t_{[22]}$ = 2.15, p = .043) (Figure 5b).

Analysis of cage control mice in the OF apparatus revealed no differences between uninfected and infected groups on percent time spent in the center ($t_{[17]}$ = .258, p = .799) or the number of entries into the center ($t_{[17]}$ = .928, p = .367) (Figure 5c). By contrast, mice living in the outdoor enclosure exhibited significant differences on percent of time spent in center suggesting that the infected outdoor mice were more anxious than the uninfected outdoor mice (percent of time in center: $t_{(22)}$ = 2.36, p = .027; entries into center: $t_{(22)}$ = 1.64, p = .11) (Figure 5d). Neither cage control nor outdoor mice exhibited uninfected versus infected differences in the total distance traveled in the OF (cage control uninfected: 4,051 $\pm$ 413.1 cm, infected: 3,462 $\pm$ 178.4 cm, $t_{(17)}$ = 1.54, p = .14; outdoor uninfected: 3,767 $\pm$ 410.8 cm, infected: 3,489 $\pm$ 329.3 cm, $t_{(22)}$ = .53, p = .60), suggesting that the difference in anxiety measures observed in the outdoor uninfected versus infected was not due to a difference in overall activity levels.

No significant correlations were observed between cognitive behavior (DRs in the OL test) and anxiety-like behavior (percent in center in the OF) in any of the groups (cage control uninfected: r = .11, p = .86; cage control infected: r = –.064, p = .84; outdoor enclosure uninfected: r = –.18, p = .64; outdoor enclosure infected: r = .33, p = .30). Furthermore, anxiety-like behavior (percent in center in the OF) did not correlate with investigation times in the OL test (cage control uninfected: r = –.57, p = .31; cage control infected: r = .28, p = .36; outdoor enclosure uninfected: r = –.66, p = .051; outdoor enclosure infected: r = .013, p = .97). There were also no significant correlations between anxiety-like behavior (percent in center in the OF) and any of the ventral microglial measures (CD68 aggregates [cage control uninfected: r = –.65, p = .23; cage control infected: r = –.59, p = .13; outdoor enclosure uninfected: r = –.16, p = .72; outdoor enclosure infected: r = –.54; p = .34], number of microglia [cage control uninfected: r = –.61, p = .28; cage control infected: r = .22, p = .61; outdoor enclosure uninfected: r = –.36, p = .38; outdoor enclosure infected: r = –.20, p = .75], number of microglial processes [cage control uninfected: r = .50, p = .39; cage control infected: r = –.23, p = .58; outdoor enclosure uninfected: r = –.43, p = .29; outdoor enclosure infected: r = .54, p = .34], microglial cell body area [cage control uninfected: r = .53, p = .36; cage control infected: r = .29, p = .49; outdoor enclosure uninfected: r = –.51, p = .19; outdoor enclosure infected: r = –.068, p = .91)].

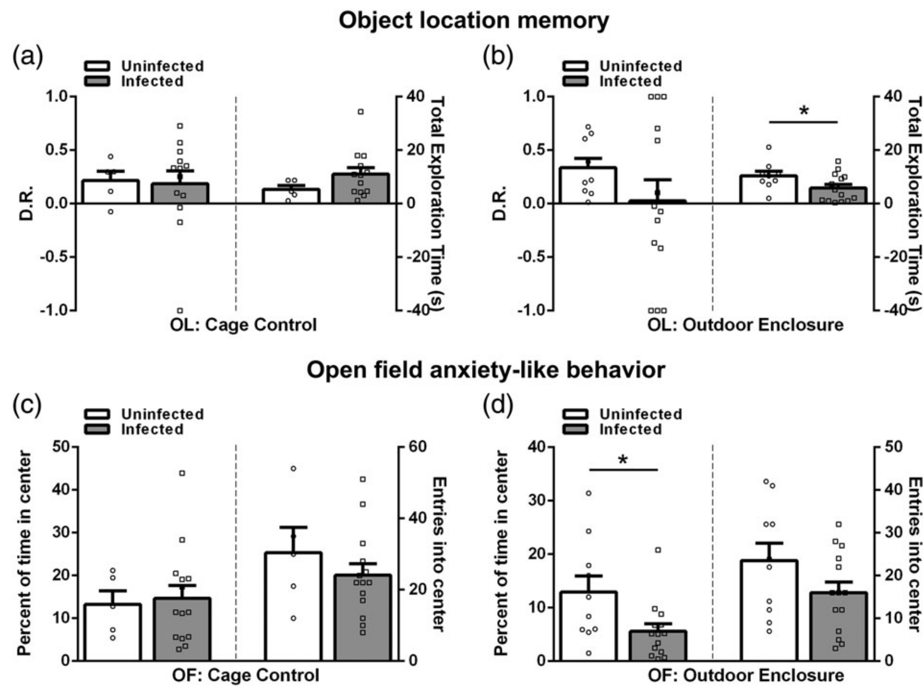

FIGURE 5 Mice with gut nematode infections living in an outdoor enclosure had increased anxiety-like behaviors. (a) Mice with nematode infections living in the cage control setting performed similarly to uninfected mice in the OL task in terms of DR and object exploration time. $N = 5$ for uninfected cage control and $n = 13$ for infected cage control. (b) Mice with parasite infections living in the outdoor enclosure spent less time exploring the objects, but did not differ in DR. $N = 9$ for uninfected outdoor enclosure and $n = 15$ for infected outdoor enclosure. $^*p = .043$. (c) Mice with gut nematode infections living in the cage control setting did not differ from uninfected mice in anxiety-like behavior in the OF test in terms of percent of time spent in center or the number of entries into center. $N = 5$ for uninfected cage control and $n = 14$ for infected cage control. (d) Mice with nematode infections living outside had increased anxiety-like behavior in the OF test in terms of time spent in the center but not in the the number of entries into the center of the OF. $N = 10$ for uninfected outdoor enclosure and $n = 14$ for infected outdoor enclosure. $^*p = .027$. Bars represent mean + SEM

4 | DISCUSSION

Our results suggest a complex pattern of effects on the hippocampus for animals living in a natural setting with and without parasitic worm gut infection. We found that living in the outdoor enclosure compared to living in standard laboratory cages increased the number of immature neurons in both the dorsal and ventral subregions of the DG, regardless of infection status. By contrast, the pattern of results for microglia activation and number revealed regional differences and some interactions between living condition and infection wherein living in a natural setting reduced microglial activation overall in the dorsal DG but only reduced microglial activation in the ventral DG of infected animals. The density of microglial cells was also sensitive to living conditions and infection status with different responses in dorsal compared to ventral DG. In the dorsal DG, setting did not affect the density of microglia but infection produced a surprising decrease in this measure. By contrast, in the ventral DG, both living conditions and infection status produced decreases in microglia density. Microglial morphology was less sensitive than microglial reactivity or density with significant effects observed only in the ventral DG and reductions in this measure in response to gut parasite infection. Analysis of behaviors associated with the hippocampus revealed no significant effects on the OL task with either living condition or infection status but significant increases in anxiety-like behavior in the OF test of mice living in the outdoor setting with parasite infection.

Our results demonstrating an increase in the number of immature neurons throughout the DG are consistent with several laboratory studies showing that living in an enriched environment stimulates adult neurogenesis (Drew et al., 2016; Kempermann et al., 1997; Tashiro et al., 2007). Since the outdoor enclosure enriched environment, with its vast richness and ample space, likely induces more variability than a laboratory controlled enriched environment, our findings emphasize the robustness and significance of enriched environment effects on adult neurogenesis in the hippocampus. It is possible that outdoor living has positive impacts on multiple stages of adult neurogenesis, such as cell proliferation, cell differentiation, and cell survival but since we did not examine cell proliferation markers in our study, for example, short-survival BrdU labeling or Ki-67, it is not clear what aspect of adult neurogenesis is altered by outdoor living. Given the larger space of the natural setting compared to the laboratory cage, as well as the evidence that mice were engaging in physical activities such as burrowing, it is possible that some of the changes we observed arose from the fact that the outdoor enclosure mice were engaging in physical exercise. It is well established that physical exercise in the laboratory robustly enhance adult neurogenesis in the hippocampus (Cooper, Moon, & van Praag, 2018; Schoenfeld, Rada, Pieruzzini, Hsueh, & Gould, 2013; van Praag, Kempermann, & Gage, 1999). Given the fact that adult neurogenesis rates decrease during the juvenile to early adulthood period in rodents (Ben Abdallah, Slomianka, Vyssotski, & Lipp, 2010), the increase in the number of immature neurons may

reflect a shift in the decreasing cell proliferation rate in the outdoor mice, although future work is needed to explore these possibilities.

The lack of any effect of parasitic worm gut infection on the number of immature neurons was surprising given that numerous studies have shown a variety of stressors, including illness induced by injection with a bacterial toxin (LPS), reduce adult neurogenesis (Bastos et al., 2008; Fujioka & Akema, 2010; Valero et al., 2014; reviewed in Opendak & Gould, 2015) and furthermore, that subsequent laboratory enriched environment living is not sufficient to counteract the negative effects of infection in a pneumococcal meningitis model (Tauber et al., 2009). When considered in this context, our findings suggest that the beneficial effects of living in the outdoor enclosure outweigh the potentially negative impact of both stressors of the outside environment, such as weather and competition for food, as well as those of infection. However, it should be noted that LPS or pneumococcal meningitis infection produce much more profound sickness behavior compared to parasitic worm infection of the gut; thus, the former infections are likely to be much more stressful. Nonetheless, previous laboratory studies examining hippocampal adult neurogenesis have shown that enriched environment living is capable of mitigating the negative effects of some prior stressors (Veena, Srikumar, Raju, & Shankaranarayana Rao, 2009) and that prior enriched environment living is capable of protecting against the negative effects of subsequent stressors (Hattori et al., 2007; Vega-Rivera et al., 2016).

Our results also suggest that living in the outdoor enclosure was sufficient to reduce microglial reactivity in the hippocampus. These findings are consistent with previous studies demonstrating that long-term laboratory enriched environment living reduces microglial reactivity, as well as those that show environmental complexity has the potential to reduce inflammatory markers in the brain (Chabry et al., 2015; Piazza et al., 2014). The ability of enriched environment living to reduce microglial reactivity in the ventral DG was most evident in the infected mice. These results are also consistent with studies demonstrating that living in enriched laboratory settings reduces viral infection-induced microglial reactivity (de Sousa et al., 2011), as well as levels of proinflammatory cytokines in the hippocampus after an immune challenge (Williamson et al., 2012). Furthermore, living in an enriched environment may promote anti-inflammatory responses of gut symbionts (Harnett & Harnett, 2010). Our microglial density results also demonstrated a decrease in the ventral DG between mice living in the outdoor enclosure and those living in laboratory cages along with a reduction in microglia density in the infected versus uninfected group living in the outdoor enclosure. Since bacterial and viral infections typically produce an increase in microglial number in the brain (Fukushima, Furube, Itoh, Nakashima, & Miyata, 2015; Hou et al., 2016), our findings from worm infected mice suggest that living in the outdoor environment not only mitigates but reverses this effect. Consistent with this, prenatal nematode infection has been shown to dampen microglial responses to subsequent immune challenges in rats (Williamson et al., 2016). It is also worth noting that there are slight, albeit not significant, reductions in mean microglial numbers in both infected groups, including those living in the laboratory as well as those living in the outdoor enclosure. One additional interpretation of our findings is that mice living in the laboratory setting have abnormally high numbers of microglia. Given that some literature suggests that gut nematodes are anti-inflammatory (Harnett & Harnett, 2010; Williamson et al., 2016), it is not that surprising that there are greater differences in microglial numbers in the infected outdoor mice which have higher worm numbers and increased worm biomass compared to infected mice living in laboratory cages (Leung et al., 2018).

Our findings regarding behaviors associated with the hippocampus in mice living in either setting, with and without parasite infection, were unexpected. Despite the fact that stress in general and infection more specifically have been shown to impair cognition (Lee et al., 2008; Valero et al., 2014) and increase anxiety (Bassi et al., 2012; Bercik et al., 2010; Savignac et al., 2016), we found no changes in OL behavior. However, our results observed when comparing uninfected to infected mice living in the outdoor enclosure demonstrated an increase in anxiety-like behavior in the infected compared to uninfected groups. The results of the OL tests of the outdoor enclosure mice were less conclusive. There was no significant difference in DR, a measure of OL memory, although the mean value was considerably lower in the infected compared to uninfected groups. There were also no significant correlations between our anxiety measures and DRs in the OL test in any of the groups. One difference that was statistically evident between the uninfected and infected groups was the increase in variability observed in the infected group. This increased variability may reflect individual differences in worm burden, response to the stress of infection, transport, or interactions among these factors. While unlikely given the lack of correlation between time spent in the outdoor enclosure and behavioral scores, it is also possible that this variability may reflect the range of days spent in the outdoor enclosure setting. However, there was a difference between uninfected and infected groups with regard to time spent exploring the objects; infected mice spent less time exploring than uninfected mice. While this difference may further reflect an increase in anxiety-like behavior, we did not find any significant correlations between OF behavior and investigation times.

Because the mice from the outdoor enclosure were captured and transported prior to behavioral testing, we did not directly compare results from those groups to mice living in the cage control setting. Nonetheless, it is evident looking across the two datasets that uninfected mice living in either setting did not deviate drastically from one another in terms of behavior. The lack of a substantial change in behavior in the uninfected mice living in the two settings may be attributable to the added stress experienced by the mice living in the outdoor enclosure. Additional studies designed to provide comparable capture and transport stress prior to behavioral testing for both groups would be needed to test this possibility directly.

The changes we observed in microglial measures between infected and uninfected mice living in the outdoor enclosure may be relevant to the differences in anxiety-like behavior detected between these groups. Since the ventral hippocampus has been directly tied to anxiety regulation (reviewed in Fanselow & Dong, 2010), changes in this area may be particularly relevant. While the available literature is limited, in general it appears that increased microglial reactivity is associated with increased anxiety and some studies have provided evidence to suggest a potential causal link between these two findings (McKim et al., 2018; Sun et al., 2016). Our results, however, do not

support this association, in that increased microglial reactivity was not coincident with increased anxiety. It is important to emphasize that the differences we observed in anxiety-like behavior might reflect changes in brain regions other than or in addition to the ventral hippocampus, including the medial prefrontal cortex and amygdala (Adhikari, Topiwala, & Gordon, 2010; Tye et al., 2011). The extent to which our findings on anxiety-like behavior, as well as microglial changes, are causally linked to the specific experiences in the outdoor enclosure, remains to be determined.

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