Supplemental Material

Materials and methods

Subcolony establishment and husbandry

Minor workers of known age were generated by establishing subcolonies of 40-70 pupae that were developmentally within 72 hours of eclosion, as identified from their yellow pigmentation. These pupae were reared with the queen, brood and approximately 150 additional workers of mixed age and subcaste demography, comprised of 5-10% major workers, within the range exhibited in natural populations [1,2]. These non-focal ants were identified by the removal of all or a portion of one leg. Once healed, they were able to normally move and interact socially. This method allowed us to distinguish focal workers without producing potential artifacts associated with marking individuals whose behavioral performance was to be assessed in bioassays. Pupae were removed every 7-10 days to prevent additional unmarked ants from eclosing; dead ants were removed and replaced with marked minor and major workers three times per week. All colonies were fed approximately every other day with 1M sugar water and a mix of protein (scrambled eggs, mealworms, earthworms or fruit flies). Subcolonies for all experiments were created from healthy, freshly collected parent colonies. Assays drew workers from 6-16 subcolonies, which were derived from up to 15 different parent colonies. Three parent colonies were used to create more than one subcolony over the course of the study (two were used twice, one was used 3 times). In all cases, colonies were allowed to recover for a minimum of 3 months before establishment of a new subcolony using new brood.

Minors of four age groups (20-22, 45-47, 95-97 and 120-122 days, of a 140-day maximum laboratory lifespan) were selected for behavioral analyses. Behavioral maturity is attained at approximately 20 days, when minor workers begin to leave the nest to forage but retain their ability to effectively perform other inside nest tasks [3,4]. If changes are similarly paced beyond maturity, we

expected to identify differences between 20 and 45-day old minors. To detect effects of senescence, two age points at 68% and 85% of the minor worker lifespan were selected. By selecting ages that represent the majority of the minor worker lifespan in the lab, which may be considerably longer than the lifespan of minors facing natural sources of extrinsic mortality, we feel confident we are able to measure senescence-associated behavioral and neuroanatomical changes that accurately represent lifespan patterns.

Behavioral assays

Nursing effort

Brood care criteria

Behaviors scored during brood-care assays were selected to encompass the breadth of minor worker behavioral responses to larvae. Workers oriented toward larvae within one-half of a worker body length (approach), which in some instances led to an act of brood care. Carrying brood was straightforward to observe, and in some cases workers placed one larva next to another (brood piling). However, as the viewing angle prevented direct observation of worker mouthparts, we used the position of the worker's body axis and antennae to identify some nursing behaviors. Consistent with findings in *Solenopsis invicta* [5], feeding larvae in *P. dentata* is often characterized by an antero-posterior body positioning (worker and larva heads in opposite directions) with antennae pointed inwards toward the larval mouthparts. Therefore, feeding was scored when a worker assumed an antero-posterior position in respect to a larva, with antennae inclined inwards toward the larval mouthparts. Workers were also observed providing care to larvae without assuming this posture. This likely involved grooming or licking the larva, but as we could not observe maxillary palps, we conservatively recorded this behavior as "unspecified brood-directed behavior." These metrics allowed us to assess sensorimotor functions in

the context of nursing behavior. Brood care in *P. dentata* is driven by olfactory cues [3], which may be integrated with tactile and/or contact chemosensory input [5], leading to performance of brood-care acts. Manipulation of delicate brood items requires fine motor coordination; minors are readily observed handling minute eggs and microlarvae without damage. Thus brood care, like foraging, involves an integration of multiple sensory modalities leading to task performance decisions requiring refined motor outputs.

Trail-following

Assay methods

Minors were anesthetized on ice and placed in the center of a 5.1 cm diameter circular trail. The trail extract was produced by removing the poison gland from the gaster with fine forceps and homogenizing glands in 100% ethanol (10 µl/gland). Serial dilutions were generated from this stock solution at 0.1 and 0.01 concentrations, and all trail extracts were prepared fresh daily and stored on ice. Preliminary analyses indicated that trails made with a 0.001 pheromone dilution induced response indistinguishable from ethanol controls, to which the ants did not respond positively or aversively. Therefore, we selected 0.01 glands per trail as our lowest concentration for our assessments of trail following. The trail pheromone extract was dispensed using a 10 µl blunt-tipped Hamilton® syringe (Microliter™ #701) onto Whatman™ filter paper (#5 qualitative, 90mm diameter, #1005-090) traced over a compass-drawn pencil trail. The syringe and arena were rinsed with 100% ethanol between all trials to prevent contamination across replicates.

Trail-following criteria

Trail-following in ants uses tropotactic orientation along a trail axis involving simultaneous spatial comparisons of pheromone samples [6] taken by antennal chemoreceptors [7]. Workers maintain

their position within the active space of a trail substance deposited on the substrate by moving the paired antennae to sample the concentration gradient of the active space of the pheromone using receptors on the funicular antennal segments (6). They make compensatory movements to adjust the position of the body axis as they orient along the chemical trail [6]. Trail pheromones are produced in specialized glands in amounts ranging from nano- to picograms and deposited on a trail; workers are able to perceive these chemicals at very low concentrations [8]. Worker responsiveness to trail pheromone, which is evident in the initiation of following and the continuity and accuracy of trail orientation, is therefore a measure of sensory perception, higher-order processing, and the neural genesis of adaptive motor outputs, all of which could be affected by senescence and chronological age. Information is transduced by chemoreceptors, and neural circuitry in the antennal lobe, mushroom body, and central complex, modulated by serotonin [9], controls following behavior. The neural circuits responsible for generating trail following could integrate movement of antennae, modify body orientation, control motion velocity, and thus coordinate locomotion.

We predicted that trail-following ability would decline in older ants and be reflected in lower accuracy, attraction to the trail, and following efficiency. These overall patterns could be reflected in lower maximum durations and distances for individual trail-following bouts. Furthermore, if older ants are more sensitive to trail pheromone, we expect the accuracy of their trail following and trail following durations and distances to be independent of trail-pheromone concentration. Younger workers, in contrast, might show longer following distances and durations within the annulus in response to trails of lower pheromone concentration. Locomotor deficits potentially resulting from senescence would be reflected in lower durations and distances within the active space.

All metrics represent measures of sensorimotor function within the estimated active space of the trail pheromone. Measurements outside the annulus were excluded to avoid confounding effects of

overall locomotion and escape attempts. Mean deviation from the trail axis was used to estimate following accuracy: large deviations from the center of the pheromone active space were interpreted as less-accurate orientation. Trail-following duration serves as a measure of both sensory and motor components: workers that spend more time within the active space likely continuously receive olfactory input that is consistently processed into motor outputs. Likewise, following distance also is influenced by sensory inputs, processing rates, and locomotion. A single trail-following bout occurred when an ant crossed into the annulus and ended when she exited the active space. We used this measurement to calculate the mean, maximum distance and duration of each bout to determine whether aging *P. dentata* minor workers follow trails with greater or less continuity.

Phototaxis

Phototaxis was recorded to assess age-related changes in responsiveness to light, which is significant in the initiation of extranidal task performance [10] and may be cued to a circadian clock that schedules activity rhythms and could show age-related decline in function [11,12]. Workers were placed with featherweight forceps into an isolation dish for 2 minutes prior to start of the assay. Ants were then transferred to the base of a tripartite maze: an opaque Y-shaped tunnel (each arm 80mm in length and 15mm in diameter) with an opaque cover to block extraneous light sources. A 15 mm diameter hole at the end of each arm allowed the ant to move toward either the bright or dimly lit arm of the Y and the observer to determine when the worker had made a selection. Workers also had the option to remain within the dark portion of the test apparatus, indicating negative phototaxis, or move toward light sources of different intensity, indicating positive phototaxis with different response thresholds. An LED lamp (MikeLite 350-2) was placed over one end creating light levels at the maze exit similar to direct sunlight (bright light; approximately 97,000 lux, measured with a Milwaukee SM700 lux meter at maze

exit; [13]), whereas the exit at the opposite end was shaded similar to a heavily overcast day (dim light; 140 lux). The remainder of the maze, except for the two 15 mm exits, were kept dark by the opaque cover. The latency to reach the end of the maze and which light level was selected (bright or dim) was recorded. All workers were assigned a random number prior to conducting the assay. It was necessary to score this assay in real-time because the difference in the lighting conditions made video recording problematic. Therefore, if only one age group of workers was being tested on a given day, it was not possible for the experimenter to be blind to ant age. However, due to the unambiguous nature of the scoring of response (a worker either did or did not exit the maze), we are confident observer bias was minimized. Latency encompasses both motor and sensory components. Minors that are more active were expected to exit the maze more quickly; workers with stronger preferences for either light level should rapidly make decisions. The placement of light in the left or right arm of the maze was made haphazardly. If a worker did not exit the maze, "darkness" was recorded as the preference and latency was set at 5 minutes. After each trial, the maze was cleaned with 50% ethanol and dried before beginning the next trial. As high-risk, outside-nest activity should be associated with positive phototaxis, we expected no decline in positive phototaxis between 20 and 120 day old minors. ANOVA was used to test differences in latency and a Chi-square for phototactic decisions (decision: N=16, 15, 22, 12; latency to exit the maze: N= 13, 10, 20, 8; 9 subcolonies sampled).

Locomotory activity

We assessed overall activity level by measuring the extent of worker movement. An individual minor worker was isolated for 2 minutes prior to the start of the assay and then placed into a 34 mm diameter Petri dish with featherweight forceps. A crosshair pattern was drawn beneath the dish, dividing it into four equal quadrants. Worker movement was digitally recorded, and the total number of

quadrants entered during 5 minutes was recorded. Observers were blind to worker age. A one-way ANOVA was used to compare activity over the four age groups assayed (N= 63, 36, 39, 22; 16 subcolonies sampled).

TUNEL

TUNEL assay

Brains were dissected in ice-cold phosphate buffered saline (PBS, pH=7.4) and placed in 4% paraformaldehyde in PBS solution stored on ice. Brains were then fixed at room temperature for 20 minutes. To end fixation, brains were rinsed one time in PBS and then washed 2 x 20 minutes in PBS. Brains were permeabilized with a freshly prepared solution of 0.1% (g/ml) sodium citrate in 0.1% Triton-X PBS (PBST) for 5 minutes. Brains were then rinsed 1 x in PBS and washed 2 x 20 min in PBS. TUNEL reaction mixtures were prepared immediately before use according to manufacturer instructions (Roche *In Situ* Cell Death Detection Kit, Fluorescein, Cat. no. 11 684 795 910) and kept on ice in the dark. The final PBS wash was removed with a Kimwipe™ and brains were added to appropriate reaction mixture tubes. All tubes were incubated in a 37°C water bath in the dark for 1 hour. The TUNEL reaction was ended by rinsing with PBS followed by 2 x 20 min PBS washes. Brains were then mounted in 5% agarose and sectioned at 100 μm on a vibrating microtom (Vibratome® 1000), sections were added to wells with PBS. Tissue sections were either stored overnight in PBS at 4°C or DAPI added (as VECTASHIELD® or NucBlue® for 30 minutes). Sections were rinsed in PBS and incubated overnight in VECTASHIELD® or 60% glycerol. If glycerol was used, brains were transferred to 80% glycerol the following morning before being mounted on slides and cover-slipped.

Brain Volume Calculations

Image stacks were imported into FIJI [14], and a composite of each physical slice was created in TRAKEM2 [15]. Regions of the rind (mushroom body, antennal lobe, optic lobe, central complex, subesophageal zone, and the remainder of the central brain) were measured. Unlike neuropil, there are no distinct divisions between rind regions. To ensure consistency between samples, a single observer measured volumes for all brain regions for 3 brains of each age group (20 and 95 days). Volume variance was similar to a random subsample of three brains using neuropil data from Muscedere et al. [16]. The means of neuropil subsamples differed on average 6.3% from the mean of all brains (n=10). The mean values of rind measurements were then used to estimate cell number for other brains. Brain regions that were incompletely represented in sections were measured individually to determine volume. All apoptosis and brain measurements were performed blind to the age of the ant.

Optical dissector

One 20-day old and one 95 day-old brain were randomly selected. A grid of 12 x 12 micron squares was placed over image stacks; squares completely contained within the rind were noted and boxes were selected at random. The number of boxes was selected so that at least 75 cells were counted per region per brain. A guard zone of 4 microns was used to avoid cutting artifacts. Because most cells were represented in at least two 2 µm optical sections (i.e., were more than 2 µm in depth), we used 4 µm as our "section" depth. Every other 4-µm section was counted. Standard rules were used to prevent over-counting. In each section to be counted the optical section 4 microns above was used as a "look-up" section. Cell bodies found in the counting section but not in the look-up section were counted; those found in both sections were excluded. Cells that fell within the counting square or touched the top and right edges were counted, whereas those that touched the bottom and left edges were excluded. For the central body, the cell body region was too small to allow adequate subsampling, thus all cells were

directly counted. Estimated cell number/brain region was calculated using volumes and counts using the equation:

$N = \overline{N}_v * V_r$

where

N = total estimated cell number for tissue/region

N_v = Local cell density for one box = cells counted * correction for skipped sections / box volume = (total count * 2)/(12 * 12 * h microns)

$\overline{N}_v$ = mean of individual N_vs for a given region

V_r = volume of the region

h = height of local counting box

Quantification of microglomeruli in the mushroom body lip region

Immunohistochemistry

Brains were dissected in ice cold PBS (pH 7.4), fixed overnight at 4°C in 4% paraformaldehyde in PBS, then rinsed 3 times in PBS. Brains were sectioned at 100 μm on a Vibratome® 1000 as described for TUNEL. Sections were permeablized in 2% Triton-X-PBS (PBST), washed in 0.2% PBST, and then blocked in 2% normal goat serum in 0.2% PBST for one hour. Slices were then incubated over 3 or 4 nights in Alexa-fluor488- or fluorescein-conjugated phalloidin (Invitrogen Inc.) and synapsin primary antibody (SYNORF, anti-mouse, Developmental Studies Hybridoma Bank). Sections were then washed 3 times in PBS and incubated overnight with Alexa Fluor 568-conjugated goat anti-mouse secondary antibody (Invitrogen, Inc.). Tissue was washed and then incubated in 60% glycerol overnight at room temperature. The following day, sections were washed in 80% glycerol for 30 minutes and then mounted on slides with coverslips, and were imaged within 3-4 days.

Microglomeruli quantification

MG density was calculated for two 400 μm diameter circles per calyx. To standardize sampling, the first circle was placed distally in the innermost upper corner of the lip, one MG width away from the neuropil edge. The second circle was placed touching the first in a diagonal position. Visible synapsin-immunoreactive puncta were counted using the manual cell counter function in ImageJ, blind to worker age. If the circle crossed any part of the synapsin-immunoreactive center of the MG, that MG was not counted. If possible, 8 circles were counted for one hemisphere (2 proximal and 2 distal circles per calyx, 2 calyces per hemisphere). When both hemispheres were counted or 4 circles counted per calyx, counts to be used in analysis were selected randomly. Because no differences in MG number were found between proximal and distal circles, these counts were averaged per calyx.

Quantification of biogenic amines

HPLC system and sample processing

The HPLC system was made by ESA (now Dionex Inc.) and composed of a model 584 pump, MD-150 (3 x 150mm) reversed-phase analytical column, a 5011A dual-channel coulometric analytical cell or a 5014B microdialysis cell, and a Coulochem III electrochemical detector. Brains were dissected in ice-cold HEPES-buffered saline (150 mM NaCl, 5mM KCl, 5mM $CaCl_2$, 25 mM sucrose, 10mM HEPES). Excess buffer was removed carefully with a Kimwipe™ and brains were homogenized in 55μl of mobile phase. Mobile phase was prepared using a formulation optimized for *Pheidole* brains [9]: 50mM citrate/acetate buffer, 1.4 mM sodium dodecyl sulfate, 0.01% triethylamine, and 24% acetonitrile in MilliQ water. Brains were kept on ice prior to measurement, centrifuged at 15,000 rmp at 2 ºC, and 50 μl of supernatent injected into a Rheodyne 975i manual injector. Electrode potentials were set for

channels 1 and 2 respectively at -125 and 225 mv for the 5011a cell and -75 and 125 mv for the 5014b cell. 5HT and DA were detected on channel 2.

Results

Phototaxis and activity level

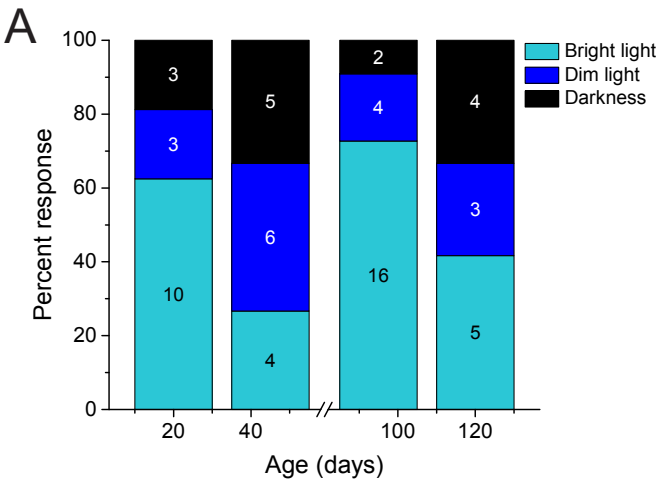
Phototaxis

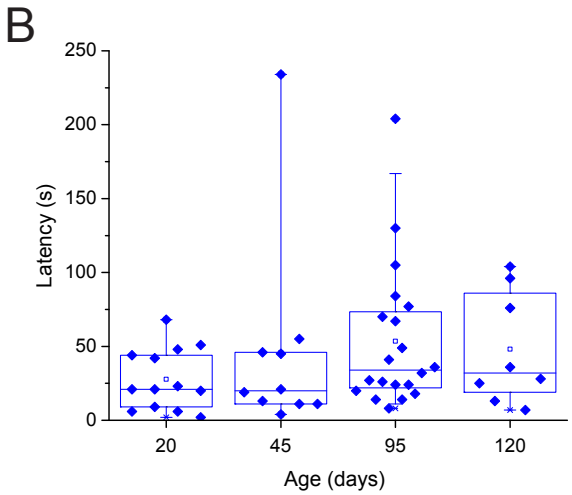
Responsiveness to light did not change in minor workers of 20, 45, 95, and 120 days of age when allowed to choose between bright light (~97,000 lux), dim light (~140 lux), or remaining in the dark (N=16, 15, 22, 12, χ_6^2=9.86, p=0.131; Fig. S1A; power = 0.875 and 0.393 for 0.5 and 0.3 effect sizes). Approximately two-thirds of workers responded positively to the two light conditions, thus their ability to depart from the dark confines of the nest to perform outside-nest tasks such as foraging appear to remain intact with increasing age. Forty-five day old minors more frequently selected dark or dim light environments, but their responsiveness did not significantly differ from minors of any other age. Minors also showed no difference in decision latency (mean ± standard error, sec: 20 days: 27.8 ± 5.76, 45 days: 45.9 ± 21.62, 95 days: 53.5 ± 10.86, 120 days: 48.1 ± 13.50; ANOVA, p=0.487, N= 13, 10, 20, 8; $F_{3,47}$=0.825, Fig. S1B; power = 0.831 and 0.377 for 0.5 and 0.3 effect sizes, respectively). Inclusion of data from minors that remained in the dark portion of the maze, and thus had the maximum latency of 300 seconds, did not change significance (N=16, 15, 22, 12, ANOVA, $F_{3,61}$=1.211, p=0.313; power = 0.912 and 0.465 for effect sizes of 0.5 and 0.3).

Activity

Activity level increased with age and reached a high asymptote for both 95 and 120 day old minors (mean ± standard error, 20 days: 40.86 ± 3.22, 45 days: 45.97 ± 3.97; 95 days: 60.54 ± 3.80; 120

days: 61.59 ± 4.97; ANOVA, $F_{3, 159}$=7.49, p<0.0001, Tukey's post hoc; 20, 45, 95, and 120 days: N= 63, 36, 39, 22; Fig S1C; power = 0.99 and 0.89 for 0.5 and 0.3 effect sizes). Qualitative observations suggest that workers with moderate to high activity rates exhibited movements typical of foragers exploring new environments or searching for food, whereas those with the highest rates of movement (>100) were similar to workers rapidly returning to the nest with food or responding to disturbance.


A
Percent response
Age (days)
Bright light
Dim light
Darkness
3
3
10
5
6
4
2
4
16
4
3
5
20
40
100
120


B
Latency (s)
Age (days)
20
45
95
120

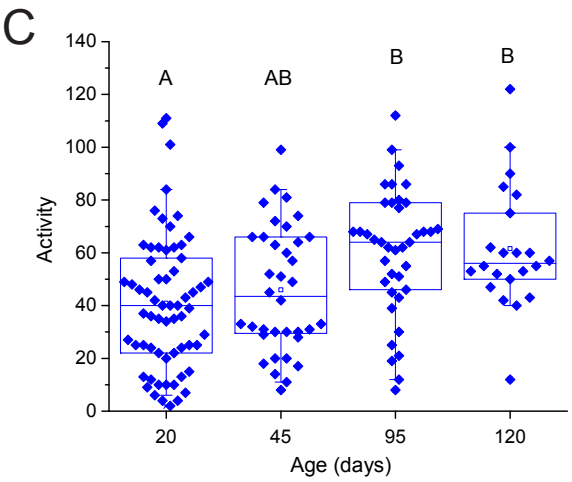

C
Activity
Age (days)
A
AB
B
B
20
45
95
120

Fig. S1. Sensorimotor function and minor worker age. **A.** Percentage response to minors presented with varying light conditions in a tripartite maze. Number of minors for each response type and age are indicated. **B.** Latency of phototactic decisions**.** Boxes indicate first and third quartiles (95% confidence intervals = whiskers). **C.** Age-related activity in minor workers (first and third quartiles indicated with boxes and 95% confidence intervals with whiskers). Letters indicate significant differences between age groups (Tukey's post hoc).

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