

Behavioral flexibility in *Wasmannia auropunctata* (Hymenoptera: Formicidae)

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Abstract

Worker division of labor is a defining trait in social insects. Many species are characterized by having behavioral flexibility where workers perform non-typical tasks for their age depending on the colony's needs. Worker division of labor and behavioral flexibility were examined in the little fire ant *Wasmannia auropunctata* (Roger, 1863), for which age-related division of labor has been found. Young workers perform nursing duties which include tending of brood and queens, and colony defense, while older workers forage. When nurses were experimentally removed from the colony, foragers were observed carrying out nursing and colony defense duties, yet when foragers were removed nurses did not forage precociously. We also administered juvenile hormone analog, methoprene, to workers. When methoprene was applied, foragers increased their nursing and defense activities while nurses became mainly idle. The behavioral flexibility of foragers of the little fire ant may be evidence of an expansion of worker's repertoires as they age; older workers can perform tasks they have already done in their life while young individuals are not capable of performing tasks ahead of time. This may be an important adaptation associated with the success of this ant as an invasive species.

Key words: behavior, flexibility, methoprene, defense, ant

Eusocial insects are characterized by having a reproductive division of labor where one or a few individuals within a colony reproduce while typically sterile workers perform other tasks (Wilson 1971, 1976). Worker tasks may include brood and queen care, foraging, and colony defense, and can be performed by particular caste or age groups. Age-based division of labor consists of young workers (i.e., nurses) that perform colony duties such as brood and queen care, and older workers (i.e., foragers) which perform duties outside of the colony that may include colony defense and foraging (Wilson 1971, 1976; Seeley 1982; Hölldobler and Wilson 1990; Jeanne 1991; Robinson 1992; Gordon 1996). The change in age-based division of labor can also be called temporal polyethism, which refers to the temporal allocation of workers through a sequence of labor groups over time (Wilson 1971, Seeley 1982, Bourke and Franks 1995).

The level of temporal polyethism varies among social insects and it is not always fixed within the colony (Wilson 1971). Depending on the colonies' needs, individuals may perform tasks that are not typical for their age. For example, honey bee (*Apis mellifera* (Linnaeus, 1758) [Hymenoptera: Apidae]) workers demonstrate behavioral flexibility, performing non-typical tasks according to the needs of the colony (McDonald and Topoff 1985,

Robinson et al. 1994, Johnson 2005). Honey bee nurses are known to forage precociously when foragers are absent, and foragers can revert to nursing when nurse populations decrease. We refer to this as the bidirectional behavioral flexibility model. This model predicts bidirectional flexibility, where foragers can revert to nursing, and nurses can accelerate to foraging.

In an ant study system, minor workers of *Pheidole dentata* (Mayr, 1886 [Hymenoptera: Formicidae]) show aged-based division of labor; younger workers perform tasks within the nest while older workers perform tasks outside the nest (Calabi and Traniello 1989). However, studies have shown that older minor workers increased their labor in response to colony needs by performing nursing tasks, while younger minor workers did not (Seid and Traniello 2006). Such results fall under the repertoire expansion model which assumes development of behavior capabilities, where individuals begin their adult life performing a limited set of tasks, and as they age their behaviors diversify to other tasks (Rosengaus and Traniello 1993, Seid and Traniello 2006). The repertoire expansion model is different from the honey bee flexibility model. The former predicts unidirectional flexibility, towards reversal. The later predicts bidirectional flexibility, towards reversal and acceleration of behaviors performed.

Behavior flexibility in the highly studied social insect *A. mellifera* has been found to be influenced by colony factors (e.g., colony demography (Huang and Robinson 1996)), environmental factors (e.g., diet and resources (Pankiw and Page 2000)), and physiological mechanisms (Toth and Robinson 2005, Ament et al. 2008) mediated by juvenile hormone (JH) levels (Robinson 1987). It has been demonstrated that JH is able to pace behavioral development of honey bees, where relatively higher amounts of JH in workers is related to forager tasks (Sullivan et al. 2000, Chang et al. 2015). Similar effects have been found in other Hymenoptera species such as ants and wasps (Gordon 1991, Giray et al. 2005, Shorter and Tibbetts 2009, Dolezal et al. 2012, Norman and Hughes 2016). High levels of JH have also been associated with colony defense mechanisms in the soldier caste of the ant *Pheidole bicarinata* (Mayr, 1870 [Hymenoptera: Formicidae]) (Wheeler and Nijhout 1981, 1983, 1984).

The little fire ant, *Wasmannia auropunctata* (Roger, 1863), is an invasive species that has spread from its native South America to every continent except Antarctica (Wetterer and Porter 2003). Due to its painful sting and its impact on agriculture and native biodiversity, it is considered a major pest throughout its range (Lubin 1984). Colonies of the little fire ant have several hundred to thousands (Le Breton et al. 2005) of small (1.2–1.5 mm) monomorphic workers and one to twelve relatively larger (5mm) queens (Wetterer and Porter 2003). Since these ants are monomorphic, we hypothesized that this species will show age related division of labor, where young individuals perform duties inside the nest (i.e., nursing) while older workers performed outside tasks (i.e., foraging).

The objective of this study was to determine whether workers of the little fire ant, *Wasmannia auropunctata*, are able to demonstrate behavioral flexibility, and classify it as either following the bidirectional flexibility model observed in honey bees or the unidirectional flexibility model (or repertoire expansion) demonstrated by *P. dentata* ants. Additionally, we aimed to test the effects of a JH analog (methoprene) on worker behavior. By experimental removal of a worker group, we examined if workers of the little fire ant expressed behavioral flexibility. If behavioral flexibility of *W. auropunctata* workers can be classified under the bidirectional behavior flexibility model, we expected that workers identified as foragers would reverse back to nursing and workers identified as nurses would accelerate to foraging tasks. Alternatively, if behavioral flexibility can be classified as unidirectional or under the behavior repertoire model, we expected that that only foragers would be observed changing tasks, while nurses would not. Moreover, if JH has a role in behavioral flexibility in *W. auropunctata*, then we expected methoprene-treated nurses to accelerate to foraging tasks as demonstrated in other species of social insects (Sullivan et al. 2000, Giray et al. 2005, Norman and Hughes, 2016).

Materials and Methods

Samples

We collected 37 *W. auropunctata* colonies from dry twigs and leaf litter in two locations at the northern karst region of the Caribbean island of Puerto Rico: an urban wetland in the city of Bayamón (18°21'17.5"N, 66°10'45.9"W), and Mata de Plátano Field Station, a Nature Preserve in the city of Arecibo (18°24'45"N, 66°43'33"W).

We separated colonies from the substrate (wood debris and leaf litter) that they were collected from and placed them in plastic 25 cm × 13 cm × 7.5 cm nest boxes, following Ortiz-Alvarado and Rivera-Marchand (2020). The nest boxes had their sides coated with Fluon

(Northern Products) to prevent ants from escaping. Additionally, we perforated the lids of each nest box with a pin to allow air exchange. The nesting area within the box consisted of a 4.5 cm² piece of thin (less than 0.5 cm thick) wood lifted 0.5 cm with a strip of clay placed along the edge of the wood. We kept boxes at conditions similar to where the ants were sampled (29°C and 80% RH). We fed ants daily with 0.5 g of feeding mixture containing agar, eggs, honey, and vitamin supplements (Hölldobler and Wilson 1994) which was placed in a feeding arena within the box approximately 10 cm from the nesting area. All colonies included in the study had one or more queens, some amount of brood (eggs, larvae, pupae), and 200–600 workers. Given that egg size is <1 mm (Wetterer and Porter 2003), instead of counting each egg in the brood, we measured the brood as the nest area (cm²) covered by the brood (see Supp Table 1 [online only] for all colonies specifics).

Age-Related Division of Labor and Behavioral Flexibility Assays

To evaluate if *W. auropunctata* has age-related division of labor, colony activity ($n = 25$) was observed during feeding periods for three consecutive days. After determining that the frequency of behaviors does not vary significantly in 5, 10, 15 and, 30-min intervals; observations were performed daily for five minutes. Workers seen tending to queens or manipulating the brood were classified as nurses. Ants feeding in the arena were classified as foragers. Observations were made under white light (Lite Mite 9 series, 475 lumens). We used cuticle color as a parameter of age category; lighter-colored individuals performing nursing were classified as younger, while darker foraging individuals were classified as older. Assignment to worker behavior by cuticle coloration was determined by multiple observers with 99% agreement. Moreover, individuals considered to have an intermediate colored cuticle were not counted during observations. We photographed nursing ($n = 15$) and foraging ($n = 15$) individuals dorsally with an Olympus SZ-14 digital camera mounted on a dissection microscope (Olympus SZX12) at a magnification of 10× under white light. Pictures were converted to a weighted RGB greyscale (Hartmann et al. 2019) and cuticle darkness at the thorax was quantified using ImageJ software (ver. 1.53 g Rasband et al. 1997–2018). The RGB greyscale varies between 0 (darkest) and 255 (lightest; Supp Fig. 1 [online only]).

Behavioral flexibility was evaluated with an experimental colony population manipulation. Colonies were fed and observed for five minutes daily on three consecutive days, and the number of workers performing nursing and foraging per colony was noted. On the third day of observations, colonies were randomly assigned a group: nurses removed ($n = 13$) or foragers removed ($n = 12$). To remove a specific caste from the colony, we separated foragers at the feeding arena from the rest of the colony and removed them with a small paint brush. For nurse-removed colonies, all workers within the colony were removed and only foragers were returned. Workers removed from the colonies ranged from 45 to 60% of the total colony population. Approximately 24 hr after manipulation, food was offered and worker activity was observed for five minutes. Food was removed at the end of each observation period. Observations were performed for four consecutive days. Worker behaviors were classified as nursing or foraging. Individuals participating in brood and queen care were considered nursing, while individuals feeding in the arena were considered foraging. Individuals outside the colony and not in the feeding arena seen performing undertaker duties (moving dead individuals) or cleaning (moving small pieces of debris) were not counted during behavior observations.

Colony Defense Behavior Assays

To evaluate colony defense behavior, we introduced a worker from a different species to the colony ($n = 6$) and measured worker response. We determined a mid-point distance of 5 cm between the main nesting area and the feeding arena for the introduced worker to be placed. After food was placed in the feeding arena, a worker from the red imported fire ant *Solenopsis invicta* (Buren, 1972 [Hymenoptera: Formicidae]) was introduced into the nest box. Defensive response was evaluated as an aggressive action (i.e., biting and/or stinging) towards the introduced individual. The period of behavioral observations lasted for 30 s after the first response. We counted the number of *W. auropunctata* workers performing colony defense behavior and classified them as young workers (light cuticle) or older workers (darker cuticle).

Colony defense flexibility was evaluated with an experimental colony population manipulation. Following the colony manipulation method we described above, we randomly assigned nurses or foragers to be removed from three colonies each. Twenty-four hours after worker removal, we repeated the colony defense assays and counted the number of workers performing colony defense behavior.

Effect of JH Analog (Methoprene) on Worker Behavior

To evaluate the effects of JH on worker behavior, we applied the JH analog methoprene to workers and observed their behaviors during feeding periods. First, we performed control observations for ten minutes as described before. After initial observations from each colony ($n = 6$), fifty individuals were collected from inside the colony performing nursing, and an additional fifty performing foraging. Colonies were randomly assigned as either treated or control. We had three colonies in the control (acetone) and three in the experimental (methoprene) groups. Individuals from the methoprene group were treated topically on the thorax with 0.5 μ l of methoprene (3.3 μ g methoprene ($\geq 98\%$ (TLC), Sigma-Aldrich) in 1 μ l acetone (ACS reagent, $\geq 99.5\%$, Sigma-Aldrich) – ca. 0.223 μ g/mg insect mass; Shorter and Tibbetts 2009, Norman and Hughes 2016). Control individuals were treated with 0.5 μ l of acetone. All individuals were marked with nail polish on their abdomen and returned to the colony. After 24 hr of treatment, we continued behavioral observations for three days. The number of marked individuals performing either nursing or foraging tasks were counted. Of the marked individuals, we noted a 2–5% mortality rate across the colonies.

Additionally, we evaluated the effects of methoprene on defense behavior. We placed twenty young and old workers in plastic cups (11.43 cm \times 11.43 cm \times 8.25 cm) coated with Fluon on the sides. We had a total of six cups from three different colonies, two cups per colony; 1 control (acetone) and 1 experimental (methoprene). In each cup we had 40 workers; 20 from each age group. Before methoprene application, defense behavior response was tested by introducing an *S. invicta* worker and measuring defensive response (as described above) for 30 s after the first response. The number of individuals by age group that showed response was counted. After a resting period of 30 min, all individuals in the cups were treated with either 0.5 μ l of methoprene or 0.5 μ l of acetone. One hour after treatment, the defense response was re-evaluated by introducing a new *S. invicta* worker. We determined the minimum time of observed behavioral effects after methoprene application during pilot studies. During these pilot studies, we measured the maximum tolerated dose and timing for observed behavioral changes. The number of individuals by age group that showed response was noted.

Statistical Analysis

Before data analysis, a Levene's test was used to verify for equality of variance, and a Shapiro–Wilk test was used to verify the normality of the data for each of the experiments. To control for colony effect, we used a generalized linear mixed model (GLMM) test to verify if the behavior count intercept and slope varied by colony. We determined that the colony effect explained little of the variation (only 0.001%).

Behavior flexibility was analyzed by the average of the three-day control observation period of individuals foraging and nursing before manipulation per colony, and compared with the daily observations after manipulations with a Repeated Measures ANOVA and a Tukey test as post-hoc method, after test assumptions were met. We analyzed colony defense behavior by comparing the number of young or old individuals that performed colony defense before and after population manipulation with a Kruskal–Wallis and Holm–Sidak test as a post-hoc, after parametric test assumptions were not met. The effect of methoprene on worker behavior was analyzed by comparing the behavior of the control and treated workers for a period of three days of observations using an RM ANOVA and a Tukey test as post-hoc method. To analyze the effect of methoprene on defense behavior we used a Fisher exact test to examine proportions of defense behavior counts between before and after methoprene treatment. Data was analyzed using the statistical program R (R Core Team 2020) v. 3.5.2 (20 December 2018) and the package glmm (generalized linear mixed models). Graphs were done in Graph Pad Prism 6.0, (GraphPad Software, La Jolla, CA).

Results

Division of Labor and Behavior Flexibility

Wasmannia auropunctata appear to exhibit age-related division of labor, with developmentally younger individuals performing nursing tasks, and older individuals performing foraging tasks. One parameter that broadly separates worker developmental age is the cuticle color. Lighter-colored individuals are younger, and darker-colored individuals are older developmentally (see Kwapich and Tschinkel 2013). In observed colonies ($n = 25$) young ants, identified by their light color (Greyscale mean = 169.30, SD = 3.69), performed nursing duties such as tending to the queen, eggs, larvae, and pupae manipulation. Foraging was performed by dark-colored (Greyscale mean = 70.67, SD = 4.43), individuals (Supp Fig. 1 [online only]; T-test: $t = 66.19$, $df = 28$, $P < 0.0001$; F-test: $F = 1.43$, $df = 14, 14$, $P = 0.50$).

To determine behavioral flexibility, we performed two worker removal experiments. In the colonies where nurses were removed (Fig. 1A), the number of individuals performing nursing after manipulation was not different from the average before manipulation ($F = 1.12$; $df = 4, 34$; $P = 0.354$). In contrast, the number of individuals performing foraging decreased significantly after manipulation ($F = 11.89$; $df = 4, 30$; $P < 0.0001$).

When foragers were removed (Fig. 1B), the number of individuals foraging decreased significantly ($F = 25.11$; $df = 4, 30$; $P < 0.0001$). Tukey post-hoc comparisons indicate that the average number of foragers before manipulation was significantly different from the number of foragers seen during the four days of the experimental observations. The amount of individuals nursing was not different during the experimental period from the observed average before forager elimination ($F = 2.70$; $df = 4, 24$; $P = 0.089$).

When colony defense behavior was evaluated by introducing an *S. invicta* worker in the nest box (Fig. 1C), most of the behavior response was carried out by young workers ($F = 34.49$; $df = 1, 14$;

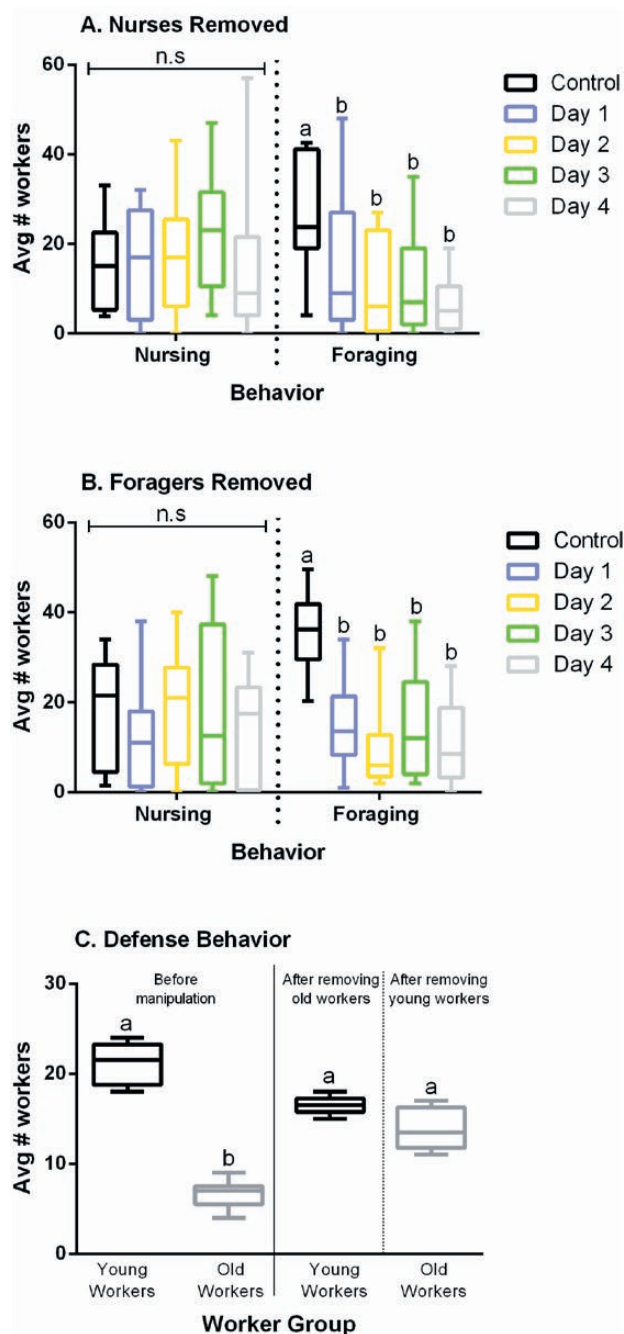


Fig. 1. Behavioral flexibility assays. A. Nurse Removal. The graph shows the average number of workers performing nursing or foraging behaviors before (control) and after nurses were removed. Total colonies $n = 13$. B. Forager Removal. The graph shows the average number of workers performing nursing or foraging behaviors before (control) and after foragers were removed. Total colony $n = 12$. C. Colony defense behavior. The graph shows the average number of workers performing colony defense response by young or old workers. Total colony $n = 6$. Boxplot represents the distribution of the average number of workers per nursing and foraging behaviors after workers were removed. Non-capitalized Arabic letters show differences between control and experimental days with a 95% confidence interval.

$P < 0.0001$). Older workers were observed returning to the nest and moving the brood out. After removing older workers, colony defense was still carried out by young workers showing no difference from the before manipulation observations ($F = 1.978$; $df = 1, 14$;

$P = 0.18$). When young workers were removed, a higher number of older workers performed colony defense behavior than observed before manipulation ($F = 72.59$; $df = 1, 14$; $P < 0.0001$).

Effects of Methoprene on Worker Behavior

Methoprene treatment showed an effect on worker behavior. When workers that were performing nursing during control observations were treated with the JH analog methoprene, the average number of individuals performing nursing was significantly lower than individuals in the control group (Fig. 2A. $F = 65.01$; $df = 1, 6$; $P < 0.0001$). Foraging behaviors in those individuals were not different in the control or treatment groups ($F = 0.22$; $df = 1, 6$; $P = 0.807$). Treated nurses remained idle inside the nest. In workers identified as foragers before methoprene treatment, foraging decreased significantly when compared to the control group (Fig. 2B. $F = 61.27$; $df = 1, 4$; $P < 0.0001$). Moreover, in treated foragers of workers nursing increased significantly ($F = 35.72$; $df = 1, 4$; $P < 0.0001$).

When we examined the effects of methoprene on colony defense behavior (Fig. 2C), before methoprene treatment, we saw similar results as previous experiments; higher number of young workers performed a defense response than older workers. However, after those workers were treated with methoprene we see the opposite; higher number of older workers performing defense response than younger workers (OR = 19.25; CI of 95% = 6.869–53.95; $P < 0.0001$). No difference was observed in the acetone-treated control group (OR = 1.47; CI of 95% = 0.43–5.01; $P = 0.76$).

Discussion

By using an age category parameter of cuticle color, workers of the little fire ant *Wasmannia auropunctata* can be classified at different developmental ages. Light-colored individuals, considered nurses, cared for queens and brood. Dark-colored individuals, considered older workers, searched for food and were seen at the feeding area. Similar associations between age and cuticle color have been described in the monomorphic ant *Acromyrmex octospinosus* ((Reich, 1793 [Hymenoptera: Formicidae] Armitage and Boomsma 2010, Norman and Hughes 2016), where a lighter cuticle color is related to nurses and darker cuticle color is related to foragers. Under typical conditions, nurses of *W. auropunctata* were never seen outside of the nest, and foragers were not seen manipulating brood.

Results from colony manipulation experiments demonstrate that the little fire ant workers have partial behavior flexibility. Older workers (i.e., foragers), which in typical colonies performed foraging, carried out nursing duties when younger workers (i.e., nurses) were absent. After young worker removal, older workers performed queen and brood care, yet foraging activity did not stop completely. The limited foraging observed, maybe due to a lack of response in foraging individuals due to there being sufficient reverted foragers performing the necessary nursing duties (Triplet and Nonacs 2004) In contrast to older worker behavior, younger workers, which typically perform nursing duties, were not observed foraging when older workers were absent. In the absence of foragers, nurses remained inside the nest and performed their typical behaviors. The limited foraging activity that was observed may have been performed by older or transitioning nurses. It is likely that these were workers who began their onset of foraging behavior within the experimental period.

During the colony defense assays, we observed that the majority of the defensive response was carried out by young workers. While

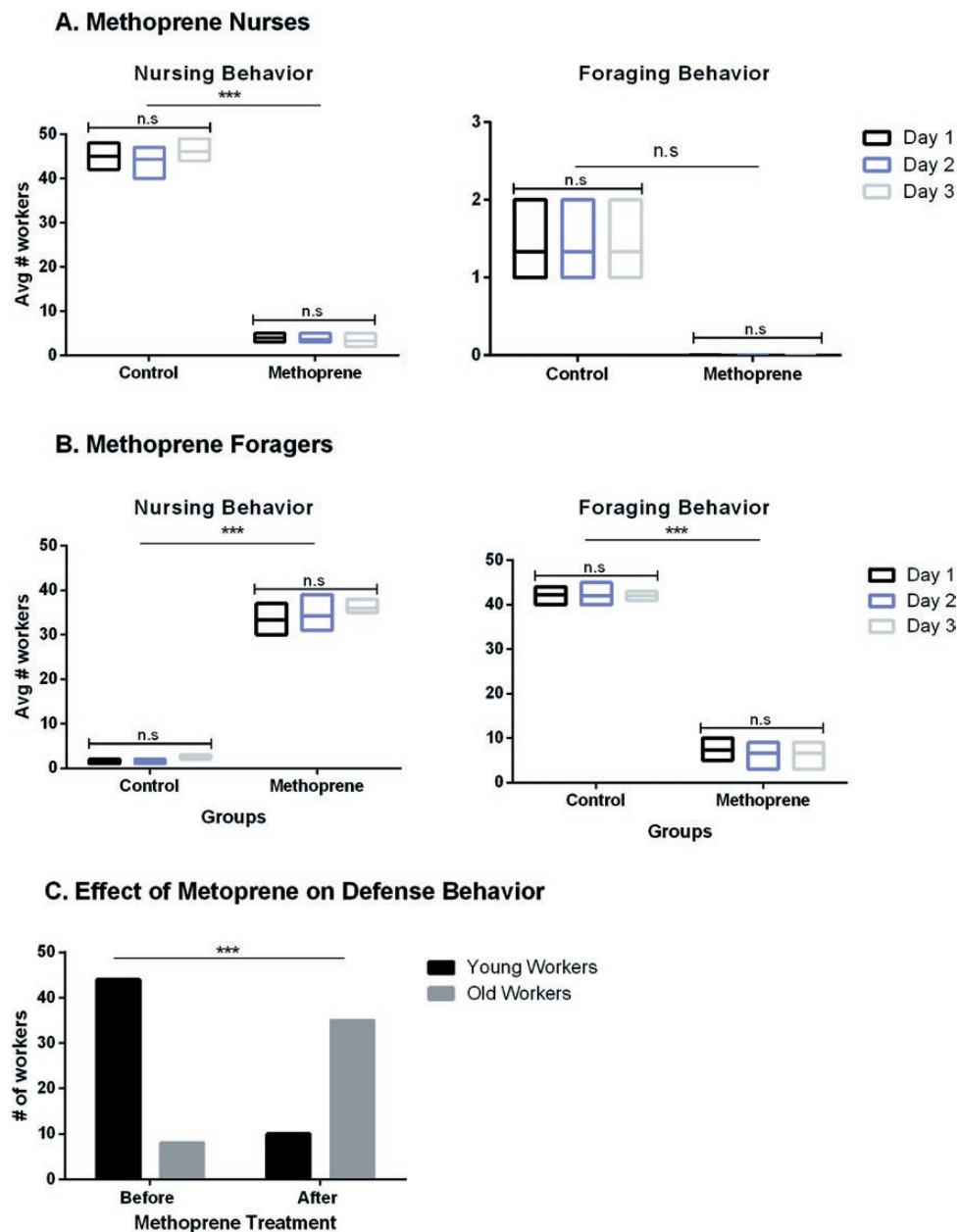


Fig. 2. Effect of methoprene in worker behavior. The graph shows the average number of workers performing nursing or foraging after methoprene treatment compared to the control group (acetone). **A.** Individuals identified as nurses in control group ($n = 150$), and methoprene treated ($n = 150$). **B.** Individuals identified as foragers in control group ($n = 150$), and methoprene treated ($n = 150$). Each plot represents the distribution of the average number of workers per nursing and foraging behaviors between control and treatment groups. *** $P < 0.0001$. Total colonies $n = 6$. **C.** Effect of methoprene on defense response. Young workers ($n = 54$), old workers ($n = 43$). *** $P < 0.0001$. Total colonies $n = 3$.

young workers displayed a defensive response, older workers were observed returning to the nest and moving out brood. This outcome in particular was surprising. Typically in social insects, older workers (eg., Honey bees; Moore et al. 1987, Breed et al. 1990) display more defensive behavior than young workers. Ants use a combination of defensive behaviors such as pheromones (Hölldobler and Wilson 1990, Vander Meer and Alonso 1998) and behavioral modifications (Hölldobler and Wilson 1998, Santos et al. 2005) among others. One possibility as to how nurses, who are inside the nest, respond quickly to intruders could be from recruitment by foragers using alarm pheromones as they enter the nest to move the brood. In colonies where older workers were removed, young workers continued

performing defense behavior. However, when young workers were removed, we observed a higher number of old workers performing defense behavior from what we had observed prior. Since older workers seem to perform a wider variety of tasks when compared to younger workers, when it comes to colony defense, we could argue that older workers are more valuable to the colony than younger workers. During colony defense, if nurses are lost, older workers can take on nursing duties, ensuring colony survival.

The capability of foragers to carry out nursing and colony defense tasks, while nurses don't seem to forage precociously, may be related to the expansion of their behavioral repertoire (Seid and Traniello 2006, Muscedere et al. 2009) or the unidirectional

behavioral flexibility model. In contrast to the bidirectional behavioral flexibility model, where foragers revert to nursing and nurses accelerate to foraging, the little fire ant seems to be only capable of reversal. Similar to other species of ants such as *P. dentata* (Seid and Traniello 2006, Muscedere et al. 2009), *W. auropunctata* might be considered an example of the repertoire expansion model.

There could be several factors that may be involved in the lack of foraging in *W. auropunctata* young ants. Nurses may depend on their age but also on their nutritional status (Toth and Robinson 2005, Dussutour et al. 2016, Ortiz-Alvarado et al. 2020). In social insects, foraging individuals tend to have lower amount of lipids than nursing individuals (O'Donnell 2001, Weeks et al. 2004, Toth et al. 2005). Some studies have also demonstrated that reversing individuals have been associated with an increase of lipid stores (Amdam et al. 2005, Bernadou et al. 2015). During the observations after forager removal, we observed young individuals feeding on eggs. Isotope and nutrient content studies of ant eggs have shown that these are rich in carbohydrates (Feldhaar et al. 2010, Melor-Ruiz 2013) which promotes higher lipid content (Weeks et al. 2004, Kunieda et al. 2006, Crumière et al. 2020). From this we can infer that young individuals might have a higher lipid reserve and hence the lack of need to forage.

Other factors that could cause a lack of foraging behavior in young ants are neurological and endocrine differences with older workers (Seid and Traniello 2005; Seid et al. 2005, 2008). Our methoprene treatment results showed an effect on behavior, likely reflecting these endocrine differences. When young workers were treated with methoprene, these individuals reduced their nursing and observed defense activity, whereas older workers treated with methoprene decreased their foraging activity while increasing their nursing activity. These results defer from our hypothesis, as we expected nurses treated with methoprene to accelerate their foraging activities as other studies have shown (Robinson 1985, Giray et al. 2005, Norman and Hughes 2016). Temporal polyethism is typically associated with physiological changes such as hormone levels (e.g., Foraging activities) being associated with an increase of JH (Robinson 1985, Huang and Robinson 1995) and lipid metabolism (e.g., loss of lipids associated with reduced nursing and increase of foraging (Toth et al. 2005, Dussutour et al. 2016). Other physiological changes are seen in ovary development (de Wilde and Beetsma 1982, Hoover et al. 2006) that typically correspond with the age of brood and queen care while ovary reabsorption corresponds to the age of foraging (McDonald and Topoff 1988, Vieira et al. 2010).

In our study, nurses treated with methoprene reduced their nursing activities and remained idle inside the nest. Moreover, we observed those nurses being cared for and groomed by other nurses. We associate those behaviors with what we previously determined as queen grooming behavior. In a previous study with *W. auropunctata* queens, it was shown that a lower expression of JH related genes is associated with foraging behaviors and higher JH related gene expression with egg laying behavior (Ortiz-Alvarado and Rivera-Marchand 2020). Taken together, methoprene treatment in nurses might be triggering a physiological response within the young worker system to lay eggs. Although it is not known if workers of the little fire ant are capable of laying eggs, this behavior has been observed in other eusocial insects (Ratnieks 1993, Iwanishi et al. 2003). JH is also known to change cuticle hydrocarbon (CHC) composition, and this composition varies depending on the task performed by the workers (de Biseau et al. 2004, Lengyel et al. 2007). CHC is involved in nest mate and species recognition in social insects (Vander Meer and Morel 1998, Stuart and Herbers 2000). The behavior observed

of young workers being groomed as if they were queens, could be related to changes in the cuticle hydrocarbons and how they are recognized by nest mates. Moreover, our results suggest that there might be a negative association between JH levels and foraging activity in the little fire ant. This is seen in foragers that reduced their foraging activity and increased their nursing activities after methoprene treatments. Interestingly, the observed defense activities also increased after treatment in older workers, a behavior that was observed to be performed by young workers.

The results of this study show differences from what was expected in other studies with other social insects. In a variety of cases, nurses were found to be able to forage precociously in the absence of foragers as demonstrated in honey bees (Robinson 1992), and the ant *P. dentata* (Calabi and Traniello 1989). The effects of methoprene were also different from what we expected. In other studies, increasing levels of JH through methoprene application led to precocious foraging in honey bees (Robinson 1987, Sullivan et al. 2000, Chang et al. 2015), species of ants (Norman and Hughes 2016) and wasps (O'Donnell and Jeanne 1993, Giray et al. 2005, Shorter and Tibbetts 2009). Although our results were different from what has been reported in social insects before, we see in general a role of JH in behavioral flexibility. Experimental approaches have tested the involvement of JH in reproduction (Brent and Vargo 2003, Lu et al. 2009), division of labor (Robinson 1987, Giray et al. 2005, Norman and Hughes 2016), and defense (Robinson 1985, Pearce et al. 2001). With the gamut of observations on JH and its role in social organization, we could argue that our results are in general in agreement with known variation in JH and plasticity in social insects.

Even if our results seem to support the idea of *W. auropunctata* workers having unidirectional behavioral flexibility, more studies are needed. There is a caveat in our experimental colonies, where the effect of age and density on behavior is confounded due to removing all the individuals of one worker type. Adding an experimental control where only part of the nurses and part of the foragers are removed would solve this. Additionally, assays where the brood is manipulated or removed to record the behaviors from workers, should also be considered. Brood pheromone is known to delay the onset of foraging on social insects and the absence of the brood promotes early foraging (Le Conte et al. 2001, Smedal et al. 2009). Therefore, we can further test the unidirectional behavioral flexibility model in the little fire ant workers. Lastly, since we are using cuticle coloration as a broad age parameter, a clear quantification of chronological cuticle color is needed to further classify little fire ant age and tasks.

The behavioral flexibility of the little fire ant may explain the success of this ant as an invasive species. An organism that arrives in a new environment may face stochastic events that can cause a population decline, especially since this ant changes colony sites frequently (personal observation; Wetterer and Porter 2003). After an event that decreases the worker population, the probability of colony growth and survival may increase by old workers investing in queen and brood care. The cost of reduced foraging may be small since the colony will only have reduced foraging capacity for a few days until some nurses age into foragers and new nurses emerge days later. High investment by young and older workers in nursing after a population decline can better benefit the colony by assuring growth during this short period.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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Author Contributions

The experiments presented in this article were conceived and designed by YOA, RFC, COA and BRM. All of the experiments were performed in Puerto Rico by YOA, RFC, COA and EDI. Data analysis was performed by YOA and COA under the supervision of BRM. The writing of this manuscript, preparation of the figures and editing was performed by all authors.

Data Availability

The original contributions presented in the study are available upon request.

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