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Original Article

Preferential food allocation to an essential worker subcaste in the invasive yellow crazy ant, Anoplolepis gracilipes (Hymenoptera: Formicidae)

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Abstract

Inside ant colonies, food distribution could be a complex behavioral process as its pattern and flow differ largely across contexts and are governed by multiple factors such as starvation level, colony size, and participating castes. However, little is known about how the food flow is regulated among worker subcastes. In the current study, we characterized the flow of dyed food between donors (foraging workers) and two in-nest worker subcastes (intra-nidal normal and physogastric workers) in the invasive yellow crazy ant, Anoplolepis gracilipes. Physogastric workers are an essential worker subcaste in A. gracilipes as they produce trophic eggs that constitute a major diet for larvae. We therefore hypothesized that physogastric workers may have a trophic advantage over intra-nidal workers as adequate nutrient intake is critical for sustainable trophic egg production. Our behavioral observations revealed that higher proportions of dyed-foodcontaining individuals were consistently found in the physogastric worker subcaste than other worker subcastes, irrespective of the macronutrient type consumed by donors. Although donors engaged in trophallaxis with intra-nidal workers more frequently than with physogastric workers, significantly higher proportions of physogastric workers were found to have dyed food in their crops 24 hours after the experiment completion. None of the larvae were detected with dyed food over the entire observation period, supporting the previous finding that food allocation to larvae in this ant species occurs primarily via trophic eggs instead of worker-to-larva trophallaxis. These results are consistent with our prediction that physogastric workers represent a "trophic center" to which food is preferentially allocated, possibly for optimization of their task performance. This study highlights the critical role of physogastric workers in the colony of this highly invasive ant and offers insights into the future development of the pest management strategy.

Key words: Food distribution, physogastric worker, trophallaxis, trophic egg.

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Introduction

Insects exhibit all levels of social organization, from solitary species, where individuals seldomly interact, to eusocial species, where individuals live together in colonies, with different subsets of individuals performing different, specialized tasks (i.e., division of labor). In eusocial insects, the high level of social organization, coupled with division of labor, necessitates a food sharing process. Food procurement is often assigned to only a small fraction of colony members (i.e., foragers), which not only satisfies their own nutritional needs but fulfills the needs of other colony members, such as queens and larvae (CASSILL & TSCHINKEL 1999, DUSSUTOUR & SIMPSON 2009, CSATA & DUSSUTOUR 2019). Hence, foragers preferentially share

their harvested food or nutrients with nestmates upon returning from a foraging journey. In ants, food is usually shared in the form of liquid and / or trophic eggs, and which of these two forms of food exchange occurs depends on food preference and / or digestive capabilities of different castes (Wheeler 1994, Meurville & LeBoeuf 2021).

In ant species that primarily rely on liquid food, foragers store the ingested liquid food in their crops (termed as "social stomach") (Hölldobler & Wilson 1990), from which the liquid food can be either moved into midgut for personal consumption or regurgitated to other nestmates through trophallaxis. Trophallaxis is defined as regurgitation, secretion, or excretion of food or materials from one



individual to another for ingestion, either through stomodeal (oral) or proctodeal (abdominal) feeding (WHEELER 1910, MEURVILLE & LEBOEUF 2021). Stomodeal trophallaxis is one of the most common types of trophallactic behaviors observed in ants, where fluids are transferred among colony members through mouth-to-mouth contacts. One of the primary functions of trophallaxis is to enable specific nutrients to reach a particular caste or colony members who are in need but unable to directly feed on the food source (WILSON 1971). Apart from the nutritional role, trophallaxis is also a major behavioral process for, among others, exchange and homogenization of cuticular hydrocarbons that contribute to nestmate recognition (BOULAY & al. 2004), building-up of collective memory that in turn facilitates the decision-making process (HAYASHI & al. 2017), sharing of gut microbiomes (LANAN & al. 2016), and transport of proteins and hormones associated with individual or colony growth (LEBOEUF & al. 2016).

Trophic eggs act as an alternative to trophallaxis as nutrients can be stored and redistributed among colony members (Yamauchi & al. 1991, Meurville & LeBoeuf 2021). Specifically, trophic eggs are packages of proteinaceous nutrients that facilitate protein flow among adults (i.e., workers and sexuals) and from adults to larvae, thus contributing to colony growth and reproduction (WHEELER 1994). Trophic eggs also represent a colony-level adaptive strategy to cope with environmental stressors such as food scarcity during the winter as trophic eggs are a longterm form of nutrient storage (e.g., being produced when needed) compared with proteinaceous food such as arthropod prey (Voss & BLUM 1987) that are easily perishable and only available for a certain period of time (LEE & al. 2017). Despite the reproductive constraints imposed on adult workers through worker policing or suppression of growth of reproductive organs (KHILA & ABOUHEIF 2010), workers in most ant species have retained functional ovaries and can lay viable, male-destined eggs and / or unfertilized, trophic eggs (HÖLLDOBLER & WILSON 1990). With the presumed costs associated with worker reproduction (e.g., reproductive workers contribute less to colony labor compared with "normal" workers), invasive ants seem less likely to possess reproductive workers (BOURKE 1988). Nevertheless, LEE & al. (2017) showed for the first time that worker reproduction occurs in an introduced population (Taiwan) of the yellow crazy ant, Anoplolepis gracilipes. LENANCKER & al. (2021) later confirmed the presence of trophic egg production in another introduced population (Australia), suggesting that this particular trait is probably common in this highly invasive ant.

Two different worker subcastes have been reported in *Anoplolepis gracilipes*: normal and physogastric workers (LEE & al. 2017). Physogastric workers can be externally differentiated from normal workers by their conspicuously enlarged gasters, which typically appear brown-whitish in color. Physogastric workers have significantly more fully-developed ovaries (as well as ovarioles) and are able to produce trophic eggs (LEE & al. 2017, LENANCKER & al. 2021). Physogastric workers have never been found to participate

in foraging and tend to stay in royal chambers with queens and larvae (LEE & al. 2017, LENANCKER & al. 2021). Observations of multiple *A. gracilipes* colonies show that larvae feed primarily on trophic eggs (LEE & al. 2017), signifying the essential role physogastric workers play in this ant species.

In ants, it has been well documented that food flow from foragers to non-foraging castes is dependent on food type or nutritional characteristics (MARKIN 1970, SORENSEN & VINSON 1981, BUCZKOWSKI & BENNETT 2009). For example, carbohydrates tend to flow preferentially to workers, while the majority of protein food is transferred to larvae and egg-laying queens (DUSSUTOUR & SIMPSON 2009, COOK & al. 2010). However, whether differential food-flow patterns occur when foragers transfer food to different worker subcastes (i.e., normal and physogastric workers) remains untested. In the current study, we used Anoplolepis gracilipes as the model system and hypothesized that food is preferentially allocated to physogastric workers because this worker subcaste may require high levels of nutrition / energy to optimize the trophic egg production. To test this hypothesis, we characterized trophallaxis behaviors between foraging workers (donors) and two worker subcastes (non-foraging, normal workers and physogastric workers) in A. gracilipes. We first examined if food (i.e., carbohydrates and proteins) flows preferentially to one worker subcaste and then if such patterns can be reflected by the frequency of trophallaxis between donors and each of the two worker subcastes in A. gracilipes. We further discuss how nutrient demand shapes the intra-caste dominance in ants and also how our results may contribute to the development of an alternative management strategy for this invasive ant.

Material and methods

Colonies

Between September 2018 and March 2019, three *Anoplolepis gracilipes* colonies were collected from three sampling sites (Nantou, Pingtung, and Taichung counties, one colony per site) in Taiwan and transferred to the lab for subsequent experimental manipulations. Ants were identified following the identification guide provided in ANTWEB (2018). All three colonies were polygynous (queen number ranging from 4 to 10), with the worker number ranging from approximately 1300 to 2500. Each colony was housed in a fluon-coated, polyethylene container ($52.7 \times 42.5 \times 13.0 \text{ cm}$) with water, 10% sucrose solution (w / w), and termites, provided ad libitum. Colonies were maintained at $25 \pm 1 \,^{\circ}\text{C}$, $60 \pm 3\%$ relative humidity and a 12:12 light:dark cycle, as were subsequent experiments and observations.

Pattern of food flow among different worker subcastes

To investigate the food-flow pattern among different worker subcastes, the flow of dyed sucrose solution and dyed peptone solution among foraging workers and non-foraging workers inside the nests was tracked. Sucrose is a disaccharide and used as carbohydrate-based food,

whereas meat peptone is protein from animal sources that have been broken down into amino acids and peptides. The peptone (Condalab, Madrid, Spain) used in this study contains approximately 70% of amino acid and traces of macrominerals, representing a suitable, protein-rich food. Workers were considered as physogastric workers if they had a visibly corpulent, brown-whitish gaster with exposed intersegmental membrane (LEE & al. 2017). Non-physogastric workers that possessed a relatively lean, brownish gaster without exposed intersegmental membrane were considered as either intra-nidal or extra-nidal, normal workers (hereafter referred to as intra-nidal and extra-nidal workers, respectively), depending on where they primarily performed their tasks (CSATA & al. 2020). Two colony fragments were isolated from each of the three original colonies (n = 6). Each colony fragment was composed of randomly selected 20 intra-nidal workers, 20 physogastric workers, one queen, and 0.02 g non-pupa brood (i.e., larvae of different instars and egg mass). Both intra-nidal workers and physogastric workers were collected within the nest harborage. Individual intra-nidal workers were marked on the dorsal surfaces of their thorax with a green paint marker (Pilot Paint Marker SC-PM, Tokyo, Japan). Each colony fragment was maintained in a smaller polyethylene container (29.0 \times 16.0 \times 9.5 cm), with its uppermost inner surface coated with a layer of fluon to prevent the ants from escaping. A 50 ml centrifuge tube that was half filled with water, plugged with cotton, and covered by a rectangular box made of bristol board ($12 \times 2 \times 2$ cm) was provided for the ants to nest as a harborage. The colony fragments were left to acclimate and then starved for 24 hours while water was supplied.

To prepare the donors, dozens of extra-nidal workers were selected from the foraging arena in the container housing the original colonies and placed in a separate container in which they were starved for 24 hours. These extra-nidal workers were then provided with either dyed sucrose solution or dyed peptone solution for at least 24 hours. A group of 30 dyed, extra-nidal workers were then introduced into the colony fragment of the same colony origin as donors and allowed to co-inhabit with potential recipients (20 intra-nidal workers, 20 physogastric workers, one queen, and larvae) for 24 hours (Fig. 1A). The dyed sucrose solution was 20% (w / w), containing 0.2% (w / w) Nile Blue A (Sigma-Aldrich, St. Louis, MO, USA - N0766 Sigma Nile Blue A), whereas the dyed peptone solution was 5% (w / w), containing 0.2% (w / w) Nile Blue A. Different concentrations were used for the two food types because the preliminary food preference test indicated that Anoplolepis gracilipes showed a high preference for the given concentrations. Nile Blue A is a highly persistent, organic dye from benzophenoxazine family that has been widely applied in biological labeling (WANG & HENDERSON 2012, MARTINEZ & HENARY 2016), and the preliminary test revealed that none of these dyed solutions produced effects on neither the survival rate of ants nor the palatability of the food. As Nile Blue A may respond to changes in solvent



Fig. 1: Schematic illustration of the experimental design in the first (A) and second (B) experiment. Numbers in the figure denote the respective numbers for each worker subcaste or donor used in both experiments.

polarity (STOCKETT & al. 2016) (e.g., fatty tissues in physogastric workers versus aqueous hemolymph in intra-nidal workers), an additional preliminary test was performed to verify whether dye visibility is altered by physiological conditions associated with worker type: The color of Nile Blue A in gaster and crop of donors and also Nile Blue A stain on the filter paper after the donors were crushed on a filter paper were observed at three observation time points (8, 16, and 24 hours). The same observation was made with both worker subcastes (intra-nidal and physogastric workers) after co-inhabitation with donors for 24 hours. The preliminary results demonstrated that the color of Nile Blue A in donors remained unchanged at the three observation time points (Figs. S1 and S2, as digital supplementary material to this article, at the journal's web pages). Sucrose- and peptone-exposed donors appeared light blue and greenish-blue in color, respectively (note that different colors here most likely result from the "cloudy nature" of peptone solution). The color of Nile Blue A in both worker subcastes was visually indistinguishable from their respective donors (Fig. S3A, D; Fig. S4A, D). All these results provided evidence that the Nile Blue A visibility was not different for the two worker types.

After completion of the experiment, the numbers of dyed-food-containing donors, intra-nidal workers, physogastric workers, and brood were counted. Individual workers or larvae were considered to contain dyed food if blue stain was detected on the filter paper on which they were crushed. Three replicates were performed for each macronutrient type, using independent colony fragments separated from different colonies (n = 3 for sucrose solution; n = 3 for peptone solution).

Trophallaxis behaviors among donors and different worker subcastes

To investigate whether the higher percentage of physogastric workers (compared with intra-nidal workers) that possessed the dyed food (see results below) was due to a high frequency of trophallaxis between donors and physogastric workers, trophallaxis behaviors between donors and different worker subcastes were recorded. The



Fig. 2: The abdomen of the donor turned blue after consuming dyed food (A); after engaging in trophallaxis with donors, dyed food was visible in both physogastric (B) and intra-nidal workers (C); none of the brood was found to have dyed food at the end of the experimental period (D). The red arrow indicates a pile of larvae of varying ages.

experimental setup was similar to that in the first experiment (Fig. 1B). Additional two colony fragments were prepared from each of the same three original colonies (n = 6), avoiding the reuse of the same ant individuals for the two experiments. Each fragment comprised 20 intra-nidal workers marked by the green paint marker, 20 physogastric workers marked by the white paint marker, one queen, and 0.02 g larvae and was maintained in another polyethylene container. The colony fragment was allowed to acclimatize and then starved for 24 hours.

After the 24-hour starvation period, an extra-nidal, dyed worker of the same colony origin (i.e., sucrose-exposed or peptone-exposed donors) was introduced into the respective colony fragment (Fig. 1B). Trophallaxis behaviors were observed under a microscope (Leica M205, Wetzlar, Germany), and the worker subcaste (intra-nidal or physogastric worker) the donor made the first trophallactic contact with was identified. Next, the numbers of all trophallactic contacts performed between the donor and all other potential recipients (20 intra-nidal workers, 20 physogastric, one queen, and larvae) within the first 30 minutes after the donor was introduced were recorded. A trophallactic contact was defined as two ant workers making mouth-to-mouth contact for more than three seconds (i.e., stomodeal trophallaxis). For each colony fragment, the experiment was repeated 10 times, each time using a new donor (i.e., a new, dyed-food-containing donor worker was not introduced into the colony fragment until the previous dyed donor worker had been observed for 30 minutes). The numbers of dyed-food-containing, intra-nidal and physogastric workers were recorded at two time points: 1) directly after the completion of the experiment and 2) 24 hours after the experiment completion. All workers and larvae were checked for the presence of dye using the method described above with two exceptions:

1) Workers were not crushed directly after completion of the experiment; instead, the color of gaster was visually inspected for the presence of dyed food, taking advantage of the fact that the gaster of *Anoplolepis gracilipes* workers is almost translucent; so the ingested food, if colored, was reliably identified by naked eyes (Fig. 2A; HAINES & HAINES 1978). 2) As the presence of dyed food in larvae could not be assessed reliably by visual inspection (especially for later instars), approximately half of the larvae was crushed directly after the completion of the experiment, and the other half was crushed 24 hours after the experiment completion. Three replicates were performed for each macronutrient type, using independent colony fragments separated from different colonies (n = 3 for sucrose solution; n = 3 for peptone solution).

Statistical analysis

For both experiments, the percentages of dyed-foodcontaining recipients were compared using generalized models with binomial error. The percentages were considered as response variables and weighted by the sample size. Experimental replications were considered as blocks in the models. For each of the examinations, different combinations or categories in the experiments were considered as the fixed effect: 1) In the first experiment, different combinations of worker subcastes (intra-nidal and physogastric workers) and food type (5% peptone solution and 20% sucrose solution) were considered as fixed effect. 2) In the second experiment, data were analyzed separately for each food type. Different combinations of worker subcastes (intra-nidal and physogastric workers) and observation time points (directly after the experiment completion and 24 hours after the experiment completion) were considered as fixed effect. The significance of the fixed effect was examined using the likelihood-ratio test by comparing the model without the fixed effect term. The post hoc multiple comparison was conducted with Tukey's all pairwise comparison test. Similarly, the percentages of dyed-food-containing donors (i.e., in the first experiment) were also compared using generalized models with binomial error, where different food types were considered as the fixed effect.

In addition, for the second experiment, difference in number of observed trophallaxis (i.e., number of observed trophallaxis with physogastric workers minus number of observed trophallaxis with intra-nidal workers) was tested by paired t-tests for each food type. All of the statistical analyses were performed using the program R (R CORE TEAM 2021), with basic functions from the "multicomp" package.

Results

Pattern of food flow among different worker subcastes

For the colony fragments with donors fed on 20% sucrose solution, a significantly higher proportion of physogastric workers (mean = $98.3 \pm 2.9\%$) received blue-dyed



Fig. 3: Box and whisker plot illustrating the percentage of dyed-food-containing ants after 24 hours co-inhabitation with donors fed on dyed food, containing different macronutrients, that is, 5% peptone solution (grey bars) and 20% sucrose solution (white bars). Horizontal bars are median values, and boxes indicate the lower and upper quartiles of the data sets. Different letters above box plots denote significant differences (p < 0.05) among different combinations of worker subcastes and food types (Tukey's all pairwise comparison test). *** indicate significant differences among different food types, p < 0.001.

food compared with intra-nidal workers (mean = $16.7 \pm 7.6\%$) (X² = 182.08; df = 3; p < 0.001) (Fig. 2B, C; Fig. 3). A similar pattern was observed in the colony fragments with donors fed on 5% peptone solution: There was a significantly higher proportion of physogastric workers (mean = $78.3 \pm 12.6\%$) found to have dyed food compared with intra-nidal workers (mean = $5.0 \pm 5.0\%$) (Fig. 3).

There was a significantly higher proportion of donors remaining blue in color when fed on 20% sucrose solution (mean = 33.0 \pm 3.0%) compared with those fed on 5% peptone solution (mean = 7.7 \pm 4.0%) (X² = 19.14; df = 1; p < 0.001) (Fig. 3), suggesting that foraging workers tend to retain carbohydrate-based food. None of the larvae (i.e., 0.02 g) was found to contain dyed food as there was no observable blue stain on the filter paper after the larvae were crushed. This suggests that neither 20% sucrose solution nor 5% peptone solution was fed by the larvae (Fig. 2D).

Trophallaxis frequency

For the colony fragments with sugar-exposed donors, donors tended to make the first trophallactic contact with intra-nidal workers (67% of donor's first trophallactic contacts): The average and standard deviation from three replicates showed that 6.7 ± 3.2 of the 10 donors first engaged in trophallaxis with intra-nidal workers, while 3.3 ± 3.2 of 10 donors first engaged with physogastric workers. A similar pattern was observed in the colony fragments with peptone-exposed donors: Donors tended to make the first trophallactic contact with intra-nidal workers; 67% of the first trophallactic interactions observed occurred between donors and intra-nidal workers. The average and standard deviation from three replicates showed that 6.7 ± 2.3 of 10 donors first engaged in trophallaxis with intra-nidal workers, while 3.3 ± 2.3 of 10 donors first engaged in trophallaxis with intra-nidal workers, while 3.3 ± 2.3 of 10 donors first engaged in trophallaxis with intra-nidal workers, while 3.3 ± 2.3 of 10 donors first engaged in trophallaxis with intra-nidal workers, while 3.3 ± 2.3 of 10 donors first engaged in trophallaxis with intra-nidal workers, while 3.3 ± 2.3 of 10 donors first engaged in trophallaxis with intra-nidal workers, while 3.3 ± 2.3 of 10 donors first engaged in



Fig. 4: Frequency histogram of the differences between number of observed trophallaxis among two worker subcastes (i.e., number of observed trophallaxis with physogastric workers minus number of observed trophallaxis with intra-nidal workers). Data were analyzed with paired t-tests (significant difference if p < 0.05).



Fig. 5: A dyed-food-containing donor was engaging in trophallaxis with a physogastric worker while surrounded by two other soliciting workers (A). A donor that completely lost the blue color in the crop directly after the end of the 30-minute observation period (B).

trophallaxis with physogastric workers. For both macronutrient types, the differences between number of observed trophallaxis among two subcastes (i.e., the number of observed trophallaxis with physogastric workers minus the number of observed trophallaxis with intra-nidal workers)



Fig. 6: Box and whisker plot illustrating the percentage of dyed-food-containing ants directly after the experiment completion and 24 hours after the experiment completion for both macronutrient types, that is, 5% peptone solution (grey bars) and 20% sucrose solution (white bars). Horizontal bars are median values, and boxes indicate the lower and upper quartiles of the data sets. Different letters within the same macronutrient type indicate significant differences (p < 0.05) among different combinations of worker subcastes and observation time points (Tukey's all pairwise comparison test).

were depicted as a frequency histogram (Fig. 4). For colony fragments with sucrose-exposed donors, the frequency of trophallactic contacts between donors and intra-nidal workers (mean = 3.2 ± 2.0 ; range = 0 - 9) was significantly higher than that between donors and physogastric workers $(\text{mean} = 1.8 \pm 1.4; \text{range} = 0 - 5) (t = -2.75; df = 29; p < 0.05)$ (i.e., negatively skewed histogram; Fig. 4). A similar trend was observed in the colony fragments with peptone-exposed donors: The frequency of trophallactic contacts between donors and intra-nidal workers (mean = 4.5 ± 2.2 ; range = 0 - 9) was higher than that between donors and physogastric workers (mean = 3.8 ± 2.2 ; range = 0 - 8), although this difference was not statistically significant (t = -1.24; df = 29; p = 0.226) (i.e., relatively symmetric histogram; Fig. 4). During trophallaxis, dyed donors were surrounded by a retinue of recipient workers, who were actively soliciting regurgitation of food from them (Fig. 5A, Video S1). Some donors completely transferred all of the acquired dyed food (i.e., no sign of dyed food in the crop) to other members (Fig. 5B).

For both tested macronutrient types, higher proportions of physogastric workers were found to contain dyed food compared with intra-nidal workers directly after the completion of the experiment, and the difference became statistically significant 24 hours after the experiment completion (5% peptone solution: $X^2 = 21.73$, df = 3, p < 0.001; 20% sucrose solution: $X^2 = 22.68$, df = 3, p < 0.001) (Fig. 6). Directly after the completion of the experiment, 58.3 ± 5.8% of physogastric workers in the colony fragments with peptone-exposed donors contained dyed food, whereas only 36.7 ± 2.9% of intra-nidal workers did. The percentage of dyed ants slightly increased 24 hours after the experiment completion, although there was no statistically significant difference (75.0 \pm 5.0% of physogastric workers and $43.3 \pm 2.9\%$ of intra-nidal workers were found to possess dyed food). A similar pattern was observed with the colony fragments with sucrose-exposed donors: Directly after the completion of the experiment, $80.0 \pm 10.0\%$ of physogastric workers contained dved food, whereas only $58.3 \pm 17.6\%$ of intra-nidal workers did. However, the percentage of dyed ants slightly decreased 24 hours after the experiment completion, although not statistically significant between the two observation time points (78.3 \pm 10.4% of physogastric workers and 45.0 \pm 8.7% of intra-nidal workers were found to possess dyed food). For both macronutrient types, despite differences in the percentage of dyed-food-containing individuals in each worker subcaste between the two observation time points, none of the larvae was found to contain dyed food, neither directly after the completion of the experiment nor 24 hours after the experiment completion, which is consistent with the findings from the first experiment.

Discussion

We performed a series of laboratory manipulations to empirically characterize differential food allocation patterns with respect to different worker subcastes in the yellow crazy ant, Anoplolepis gracilipes. Compared with intra-nidal workers, a higher proportion of physogastric workers was found to possess dyed food directly after the end of the first experiment, irrespective of the macronutrient type consumed by donors. Although foraging workers tended to engage in trophallactic contacts with intra-nidal workers more frequently than with physogastric workers during the first 30 minutes after they were introduced, higher proportions of dyed-food-containing physogastric workers were observed directly after the completion of the experiment and 24 hours after the experiment completion. All these data suggest the occurrence of preferential food allocation to physogastric workers in A. gracilipes. Furthermore, no larvae were found to contain dyed food at the end of both experiments. Below, we discuss potential mechanisms responsible for the observed differential food allocation patterns among different subcastes and also provide insights into the pest management of this invasive ant.

Preferential allocation of food to physogastric workers

Once returning from a foraging journey, foragers share ingested food with non-foraging members inside the nest. In general, due to the varying nutritional needs of different castes, workers tend to selectively distribute the protein to larvae and queens, while carbohydrates tend to be retained by foragers or quickly propagated among other non-foraging workers (SORENSEN & VINSON 1981, CASSILL 2003, BUCZKOWSKI & BENNETT 2006). Relatively few studies have examined the food regulation by different worker subcastes inside ant colonies. SORENSEN & al. (1985) showed that intra-nidal workers (e.g., nurses) of the red imported fire ant, *Solenopsis invicta*, primarily regulated the flow of proteins and lipids inside the colony, in which the acquired nutrients were either for their own use or transferred to larvae directly. Our findings demonstrate that in *Anoplolepis gracilipes*, liquid food tends to be preferentially allocated to physogastric workers as higher proportions of dyed-food-containing individuals were consistently observed in the physogastric worker subcaste than in other subcastes.

Workers must obtain a suite of macronutrients to satisfy their own nutritional requirements and also to optimize their task performance. As active, well-developed ovaries in physogastric workers are essential in sustaining the trophic egg production in the colony, one would predict that physogastric workers may require key amounts of nutrients to maintain their "reproductive" capabilities. Our findings from both experiments provide promising support to this prediction. First, peptone solution was found in more physogastric workers than intra-nidal workers, and protein resources are expected to promote ovarian and egg development in the reproductive caste of social insects (HOOVER & al. 2006, HUMAN & al. 2007, COOK & al. 2010). Second, while protein is necessary for the growth and development of the colony, increasing evidence suggests that the consumption of carbohydrates can also increase reproductive rates and subsequently leads to a larger colony size in ants (WHEELER 1996, HELMS & VIN-SON 2008, WITTMAN & al. 2018). This may explain a higher proportion of physogastric workers found to possess sucrose solution in both experiments of the current study.

Within-colony trophallactic interactions

Nearly all Formicinae and Dolichoderinae are known to perform trophallaxis, through which exogenous nutrients and endogenous signals can be effectively distributed among colony members (MEURVILLE & LEBOEUF 2021). In general, food can be rapidly disseminated throughout the ant colony within minutes or a few hours after a substantial proportion of workers have returned from their foraging trips (MARKIN 1970, BUFFIN & al. 2009). Similar to previous findings, our study (i.e., colony fragments with sucrose-exposed donors) showed that the majority of physogastric workers ($80.0 \pm 10.0\%$) received blue-dyed food within 5 - 6 hours after the first donor was introduced, whereas only 58.3 \pm 17.6% of intra-nidal workers received blue-dyed food over the same co-inhabitation time.

One may expect a higher trophallactic contact frequency between donors and physogastric workers since there are significantly more dyed-food-containing physogastric workers than intra-nidal workers. However, our observations from the second experiment showed the opposite – that donors, in fact, engaged in trophallaxis with intra-nidal workers more frequently than with physogastric workers, irrespective of the macronutrient type consumed by the donors. Based on our observation, intra-nidal workers tend to have a relatively higher level of exploratory activity (data not shown) than physogastric workers after being isolated from their original colonies. This may subsequently lead to a higher encounter rate with donors. Consistent with earlier studies (LEE & al. 2017, LENANCKER & al. 2021), physogastric workers showed a tendency of staying in the harborage with brood and queens in both experiments, which may have substantially reduced their encounter rate with donors.

Such differential interactions between donors and different subcastes may be associated with social / organizational immunity (STROEYMEYT & al. 2014, CREMER & al. 2018). In social insects, the presence of different organizational groups within a colony and different interaction levels among these groups may contribute to the mitigation of disease spread as members performing the tasks with higher disease risk (e.g., foragers) often interact less with the most valuable colony members (e.g., the immature and reproductive individuals). Such interactions based on organizational structure would contain a disease within subgroups rather than spreading among most members of the colony, including larvae and queens (STROEYMEYT & al. 2014, CREMER & al. 2018). Reduced physical contacts between donors and physogastric workers in Anoplolepis gracilipes may represent an adaptive behavior that restricts disease transmission from workers to other essential worker subcastes such as physogastric workers. Further studies should focus on empirically testing if this behavior is reinforced when the colony is affected by disease.

What factors could possibly lead to more dyed-food-containing physogastric workers at the end of the experiment? One possibility is that donors unload (partially or fully) their crop contents to a group of recipients (i.e., intra-nidal workers), and these recipients may act as "secondary" donors whose crop contents are eventually offered to physogastric workers. Although the food dissemination pattern among all individuals was not tracked in the current study, trophallactic interactions among intra-nidal and physogastric workers were consistently observed during the experiment (Video S2). Such a trophallactic cascade is seemingly common in ants (HOWARD & TSCHINKEL 1980, BUCZKOWSKI & VANWEELDEN 2010) as well as other social insect systems (Suárez & Thorne 2000, Buczkowski & al. 2007). To address these fundamental questions, further studies are needed that include tracking food transfer to create a trophallactic network and additional critical laboratory manipulations (e.g. long-term access to food sources).

Physogastric workers as essential trophic specialists

In the present study, we found no evidence that the larvae directly receive food from workers, which is consistent to an earlier study that showed *Anoplolepis gracilipes* larvae primarily feed on trophic eggs produced by physogastric workers (LEE & al. 2017). Trophic eggs are nutritional packets covered by a less rigid chorion that allow easy storage and consumption by various ant castes, including larvae, sexuals, and adult workers (WHEELER 1994). In most ant species that rely on trophic eggs for food storage and exchange, trophic eggs are prerequisite for the growth and development of a specific caste and / or

developmental stage in the colony (CRESPI 1992, WHEELER 1994). For instance, PEETERS & al. (2013) showed that trophic eggs in the ant Crematogaster biroi are primarily consumed by older larvae as the sole food source. Our work demonstrates that trophallactic fluid feeding of larvae seems rare in A. gracilipes, regardless of the larval instar. This finding once again underscores the importance of physogastric workers as essential trophic specialists in colonies of this invasive ant. However, we cannot exclude the possibility that the amount of dyed food acquired from donors was so small that the absence of Nile A Blue stain on the filter paper is a false-negative result. Moreover, larvae are in general the "end-users" of food resources and there is a possibility that larvae receive liquid food at longer timescales (CASSILL & TSCHINKEL 1995). In other words, larvae may obtain food from donors only after a few days of co-inhabitation time.

Pest management implications

The findings in this study may have important pest management implications. Ant baits function by taking advantage of trophallaxis behavior as the bait toxicant can be effectively shared among colony members through active trophallactic interactions (STRINGER & al. 1964, WILLIAMS & al. 2001, Rust & al. 2004). A baiting program involving liquid bait should be effective in controlling Anoplolepis gracilipes because a significant amount of bait toxicant would be accumulated in physogastric workers as a result of preferential food allocation, which presumably leads to a faster mortality of this essential worker subcaste. Consequently, larval mortality can be expected due to the diminished supply of trophic eggs, significantly slowing down worker turnover rate and thus colony productivity. The fact that promising control efficacies were consistently found across multiple A. gracilipes control / eradication campaigns (LEE & YANG 2021) appears to support our prediction. Yet, a critical question remains whether solid food is processed or disseminated in the A. gracilipes colony in a similar pathway as liquid food. It is well documented that larvae, especially fourth instar larvae, are a key stage that regulates solid food flow in fire ant colonies, and such a food processing pathway is linked to the management success of this pest ant when using solid granular baits (PETRALIA & VINSON 1978, HOWARD & TSCHINKEL 1981, CASSILL & TSCHINKEL 1999). Therefore, further studies are needed to understand the dissemination pattern of solid food in this ant, which may potentially provide insights into the observed inconsistencies in the control effectiveness of different bait formulations (LEE & YANG 2021).

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