# Age-dependent physiological changes and subcaste roles in a worker dimorphic ant *Camponotus yamaokai*

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**ABSTRACT.** Some ants have morphologically distinct subcastes among workers, divided into minor workers, which are smaller, and major workers, which are larger and often have enlarged heads. Minor workers perform general tasks, while major workers often have more specialized roles, such as nest defense and nutritional storage. In addition to morphological caste differences, age plays an important role in polyethism. Generally, young workers having high fat mass become nurses, while old workers with reduced fat mass shift to foragers. However, few studies have examined the relationship between subcaste and age-dependent division of labor, and associated physiological changes. In this study, we used *Camponotus yamaokai*, which exhibits clear dimorphism among workers, to clarify the specialized roles of major workers in terms of behavior and physiology. We found that major workers rarely forage or leave the nest but instead defend the nest by plugging the nest entrance when heterospecific competitors are present in the feeding area. In histological observation of the lipid storage organ (i.e. fat body), minor workers showed a reduction in fat body size with aging, the typical of worker age-dependent changes. On the other hand, physiological changes with aging were minimal in major workers. These findings indicate that major workers of *C. yamaokai* maintain a stable, 'young' physiological state and are specialized for intranidal nest defense, rarely shifting to extranidal tasks with age.

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# INTRODUCTION

In some ant species, morphologically distinct worker subcastes exist, with larger individuals typically referred to as major workers or soldiers, while smaller ones are known as minor workers (Wilson 1953; Wilson 2003). In general, minor workers are responsible for broad, basic tasks such as nest maintenance, foraging, and brood care, while major workers are responsible for more specialized tasks such as defending nests and feeding areas, nutritional storage, and food milling. Thus, there is a division of labor among the morphologically distinct subcastes (Hölldobler & Wilson 1990; Tschinkel 1993; Yang et al. 2004; Mertl & Traniello 2009; Huang 2010; Lillico-Ouachour & Abouheif 2017). The division of labor is also age-dependent; with the shift from intranidal to extranidal tasks, such as foraging (Hölldobler & Wilson 1990). Physiological changes underlie this division of labor, particularly the loss of fat mass with aging that may lead them to the transition to a forager role (Blanchard et al. 2000; Toth & Robinson 2005; Bernadou et al. 2020).

The fat body, the main organ of nutritional storage in insects (Ricks & Vindon 1972; Elliott & Davison 1975), stores fat and has functions comparable to those of the liver and fat tissue in mammals (Dethier 1976). The fat body of adult Hymenoptera is composed of two types of cells called trophocytes and oenocytes (Locke 1984; Dean 1985). Trophocytes are the primary cells of the fat body and have large, irregular nuclei, which store materials such as proteins, fats, and carbohydrates, as well as synthesize proteins. Oenocytes are smaller than trophocytes and are located among trophocytes (Roma et al. 2006a; Roma et al. 2010). Although the function of oenocytes is not completely understood, they are known to synthesize carbohydrates, accumulate fat during starvation, and facilitate the fat metabolism in trophocytes (Gutierrez et al. 2007; Roma et al. 2010). It has been shown in Apis melifera that trophocytes shrink, and the number of oenocytes relative to the number of trophocytes increases in old individuals compared to young individuals (Strachecka et al. 2017). Since age-dependent loss of fat reserve is observed in social hymenoptera (Blanchard et al. 2000; Toth & Robinson 2005; Bernadou et al. 2020), age-dependent changes in fat body morphology may be associated with the age-dependent division of labor and worker behavioral roles. Age-dependent changes may thus be understood from the morphology of trophocyte and oenocyte cell organization.

Fat stored in ants is considered a nutritional resource that can be shared among colony members (Tschinkel 1987; Hasegawa 1993). Species with morphological subcastes often have high fat content in major workers, which is known to be important for overwintering or starvation (Wilson 1974; Hasegawa 1993; Yang 2006). In monomorphic ant species, task allocation pattern changes with age, and the amount of fat in workers generally decreases as they get older (Blanchard et al. 2000; Toth & Robinson 2005; Bernadou et al. 2020). However, the changes in stored fat may differ among subcastes in polymorphic ant species. For example, species in which major workers have a storage or defense role may not show age-dependent changes in fat. Minor workers are expected to follow the typical age-related division of labor and fat loss pattern observed in monomorphic ant species. In contrast, major workers may follow a different pattern due to their specialized roles. If major workers primarily engage in intranidal tasks such as food storage or nest entrance plugging, we hypothesize that their physiological state remains stable with age, with less fat reduction than in minor workers. However, age-dependent changes in stored fat are not well known in polymorphic ant species. Understanding these physiological changes is important for elucidating the subcaste-dependent division of labor and its relationship with fat dynamics.

In this study, we focused on Camponotus yamaokai Terayama & Satoh, 1990. This species has a distinct dimorphism in workers, with minor workers having a head width of 0.88-1.03 mm and major workers having a head width of 1.28-1.38 mm (Terayama & Satoh 1990). Unlike typical Camponotus species and closely related species, intermediate-sized media workers are extremely rare, and the body size distribution is clearly divided into two groups (Satoh 1990; Wills et al. 2018). They are polydomous, with about 200 individuals per nest, and major workers comprise about 15% of the colony (Satoh 1990). Multiple queens are considered to be functional egg layers in this species (Satoh 1990). Because of the small size of colony fragment (i.e. nests) and the absence of an incipient colony stage due to dependent colony founding (Satoh 1989; Satoh 1990), it was considered suitable for laboratory manipulation of worker composition of the nest. Although several previous studies have focused on C. yamaokai, ecological and behavioral knowledge among subcastes is still lacking (Sanada et al. 1998; Koyama et al. 2015; Ghosh & Cronin 2021; Chiyoda et al. 2023; Chiyoda et al. 2024).

In this study, we investigated the behavioral specialization of major workers in *C. yamaokai* by focusing on foraging, defense, and age-related changes in fat storage. For this purpose,

we conducted the following three experiments: (1) Behavioral observations in the laboratory to assess the foraging frequency of major workers; (2) Experimental cohabitation with other ant species to observe the defense behavior of major workers; and (3) Histological analysis to examine age-related physiological changes in fat body.

# MATERIALS AND METHODS

# Ant

Camponotus yamaokai nests within hollows of dead tree twigs or bamboo grasses. Collected colonies were brought back to the laboratory with their nests and transferred to rearing containers. The rearing case (feeding site) was a styrene square case (180 x 90 x 45 mm) with the walls coated with Fluon to prevent escape and the lid closed. Nests were made by printing PLA (polylactic acid) containers (OD 85 x 28 x 11 mm) using a stacked 3D printer, gluing a 1 mm thick linden wood board inside the nest, and covering it with a glass slide (Fig. 1). There is one nest entrance, a 4 x 2 mm rectangle. A red plastic sheet was placed over the nest to shade it from light. The inner surface of the nests was made of wood, which was close to the original nesting condition, and could be observed with a glass slide. A 2 ml micro tube with a hole in the lid, filled with water and capped with a Kimwipe (Kimberly Clark Corp., Texas, U.S.), was always kept in the rearing case as drinking water. Food consisted of cut-up frozen crickets, honey, Ant Supplement (Ant room co., Tokyo, Japan) or Pro Jelly (insect food jelly, KB Farm, Saitama, Japan) dissolved in water at 10% concentration, and flour beetle larvae (Gnatocerus

*cornutus*) twice a week. The feeding area was also cleaned of garbage and the floor was wiped clean about once a month. Temperature conditions were 25°C and light conditions were 14h light/10h dark cycle (lights on at 7:00 and off at 21:00).

### **Observation of foraging behavior**

Three colonies, collected in January, April, and September 2022 in Hachioji City, Tokyo, and reared in the laboratory, were used for this experiment in December 2022. The numbers of queens, major workers and minor workers were n=22, 18, 88 (Colony20220115), n= 4, 12, 93 (Colony20220407) and n= 3, 17, 144 (Colony 20220915) respectively. Lidless containers (125 x 166 x 78 mm) were used as feeding areas, into which ant colonies were placed with their nests. A night-vision camera (Kuman Tec, China) that enables observation in the dark was used to capture images once every 30 minutes for 10 days, including the feeding dish (40 mm diameter petri dish) in the frame. The ants were fed once every four days during the experiment. Water supply, light cycle, and temperature conditions followed the rearing protocol described previously. The number of foragers of each subcaste (i.e., major or minor) was counted from each image. We defined foragers as all individuals located outside the nest, regardless of whether they were in contact with the food source. Subcastes were clearly distinguishable based on size and head morphology. Statistical analysis was conducted using the R system for statistical computing (R Core Team 2022, version 4.2.2), and Fisher's exact was applied to compare the subcaste ratios of foragers with that of the experimental nest.



Fig. 1. Artificial nest used in the experiment.

The outer frame was created using a 3D printer, a wooden board was attached to the inside, and the nest was covered with a glass slide.



Fig. 2. Caste ratio and foraging frequency.

Foraging frequencies of major and minor workers. The difference of foraging propensity of major and minor workers is shown as the frequency bias of foraging behavior and the caste ratio in the colony. Fisher's exact test, \*\*\*: p<0.001



Fig. 3. Frequency of entrance-plugging time.

Frequencies of entrance-plugging time was compared between castes (colors) and conditions. The three distinct symbols on each line represent the three different colonies used. Experimental nests were reared in two different conditions; with heterospecific competitors (*Temnothorax congruus*, with competitors) or without competitors (no competitors). The significant effect of interaction between competitor condition and caste was detected (p < 0.001, Binominal GLMM (link = logit, . \*\*\* p<0.001, see Table 1 for detailed results), suggesting that major and minor workers responded differently to competitors exposure.



Fig. 4. Microscopic images of *C. yamaokai* fat body cells.

(A) Young minor worker (under four months old). (B) Old minor worker (over seven months old). (C) Young major worker (under four months old). (D) Old major worker (over seven months old). Nuclei are shown in green (Sytox green) and lipid droplets are shown in magenta (Nile Red). The cells indicated by blue lines are trophocytes and the cells indicated by white arrows indicate oenocytes. Fat body of young minor (A), young major (C) and old major (D) contained lipid droplets in trophocytes, but that of old minor (B) was heavily degenerated and lipid droplets were scarcely found in trophocytes.



Fig. 5. Characteristics of trophocytes and oenocytes in different castes.

(A) Lipid contents of fat body represented by lipid droplet area in optical section. Each dot represents the mean value of 100 droplets in one individual. (B) Frequency of oenocytes in fat body shown by the ratio of oenocyte number to trophocyte number. Age-dependent changes were not detected. (C) Trophocyte size shown by cross-sectional area. Major workers had larger trophocytes. (D) Oenocyte size shown by cross-sectional area. Major workers had larger oenocytes. In old minor workers, fat bodies were heavily degenerated and cellular structures could not be identified (NA). Each dot represents the mean value of 10 cells in one individual. The box plots depict the median, quartiles, and range of the data. Statistically significant differences were shown by different letters (A: Nemenyi test, p < 0.05; B, C, D: Tukey HSD, p < 0.05).

# Observation of defensive behavior of major workers against other ant species

Two colonies of *C. yamaokai* collected in April 2023 in Musashimurayama City, Tokyo and one colony collected in July 2023 in Hachioji City, Tokyo, were used for this experiment. The numbers of queens, major workers and minor workers were n = 3, 25, 163 (Colony20230405-01), n = 7, 21, 262 (Colony20230405-04) and n = 3, 48, 219 (Colony20230721-06) respectively. From each colony, an experimental colony of 85 minor workers, 15 major workers, and 1 queen was created and moved to a new nest. This composition was chosen to minimize variability in the influence of colony size and the number of major workers,

and to approximate the average proportion of subcaste composition observed in wild colonies (Satoh 1990). As a competitor, three *Temnothorax* congruus colonies were collected from Hachioji City, Tokyo, and reared in the same way as described for *C. yamaokai*. In wild, *T. congruus* inhabits the same area as *C. yamaokai* and has almost the same nesting habitat. A container without a lid (125 x 166 x 78 mm) was used as a feeding area, and each experimental colony of *C. yamaokai* was placed in it. After rearing the colony for three days to acclimate them to the environment, we began recording them with a night-vision camera. The colony was kept without competitor ants for the first three days and then *T*.

congruus colony were introduced for the next three days. Three experimental T. congruus colonies of 100 workers and one queen were prepared, and each was placed in a feeding area diagonal to the nest of C. yamaokai. The night-vision camera was positioned so that the feeding dish (40 mm diameter petri dish lid) and the entrance to the C. yamaokai nest were included in the frame, and the camera took a picture once every 30 minutes. The ants were fed once every four days. Water supply, light cycle, and temperature conditions followed the rearing protocol described previously. The number of plugging individuals was counted for each subcaste from the images. Since the identification of plugging behavior was based on images, we could not strictly distinguish whether individuals were simply passing by or deliberately staying at the nest entrance. Therefore, we operationally defined plugging as an individual close enough to contact the entrance with its antennae or head within the entrance and facing out of the nest, acknowledging that this may include some ambiguity.

A generalized linear mixed model (GLMM) was used to analyze the data to examine differences in the effects of subcaste and the presence of other ant species on the presence or absence of nest entrance plugging. We used lme4 (version 1.1-32), an R package for GLMMs, and the model was binomial distribution (link = logit). Subcastes were divided into binary (major or minor workers), and competitor conditions were also binary (no competitors or with competitors) (Bates et al. 2015). For each image, the presence or absence of plugging behavior was recorded separately for each subcaste. If at least one individual of a given subcaste was observed plugging behavior, the plugging value was coded as 1 for that subcaste at that timepoint; otherwise, it was coded as 0. Model assumptions were evaluated using the DHARMa package (Hartig 2022), and residual diagnostics indicated no substantial deviations. Data were modeled as follows:

Random effects are given as , where is the variance of the random effect of the colony. The significance level was set at P < 0.05.

# Observation and quantification of fat body cells

Six colonies collected in Tokyo (Hachioji and Musashimurayama), and Tsukuba City, Ibaraki

Prefecture, between January and October 2022 were used in this experiment. All individuals in each colony were marked with Mitsubishi paint markers in three locations on the head, thorax, and abdomen in the same color in order to determine the age of individuals. Newly hatched individuals were also marked once every one to two months. CO<sub>2</sub> gas anesthesia was used as short as possible during marking. In this study, workers who had been present since collection (>seven months old) were defined as old, and workers who had hatched under the laboratory (<four months old) were defined as young.

The ant gaster was dissected in PBS, and fat bodies were extracted. In ants, there are two forms of fat bodies, one lining the cuticle and the other around the gastrointestinal tract (Chapman 1998, Gullan and Cranston 2000), which have been reported to differ in cell size (Roma et al. 2006b). In this study, only the fat bodies lining the cuticle were used in this experiment. Fat bodies were placed in 200 µl of 4 % PFA and shaken for 20 min or placed in the refrigerator overnight. The PFA was removed, and 200 µl of PBT was added and shaken for 20 minutes. PBT was removed, washed once with 200 µl of PBS, and only PBS was removed. SYTOX® Green (SY) from Thermo Fisher Scientific was used to stain nuclei, and Nile Red (NR) from Wako was used to stain lipid droplets. A PBS solution containing 0.045 % SY and 0.5 % NR was prepared for a total volume of 200 µl per sample. This was added to the fat body cells and shaken for 20 minutes in a shaded condition. The stain was then removed, and 200 µl of PBS was added.

A Nikon confocal laser microscope C2 (Nikon, Japan) was used to observe and capture images of fat body cells in the preparation. The objective lens magnification was 20, and one or two areas were selected per sample where cells could be observed in clusters, and Z-axis stacked images of 633.99 x 633.99  $\mu$ m (1024 x 1024 pixels) were acquired.

Three parameters were measured from the acquired images: (1) the number of trophocytes and oenocytes, (2) the cross-sectional area of lipid droplets, and (3) the cross-sectional area of trophocytes and oenocytes. Fiji was used as the image analysis software (Schindelin & Frise 2012). The cell membrane and cytoplasm of fat body cells were also lightly stained by SY so that the outlines could be seen by adjusting the brightness, and cell size and other parameters could be measured. However, the quantification of (1) and (3) was not possible in old minor workers because their fat bodies were heavily degenerated (Fig. 4B). Detailed methods are as follows. (1) The cross-section with the cell cluster was selected, and the number of trophocytes and oenocytes in the image was measured. The cells cut off at the edge of the image were also included in the count. (2) The section with the most lipid droplets clearly visible was selected and binarized. The area of 100 lipid droplets was randomly measured from the image, and the average lipid droplet area was calculated. (3) The Z-axis with the largest crosssectional area of each cell was selected, and the cross-sectional area of one cell was measured. For each individual, the area of 10 trophocytes and 10 oenocytes were measured, and their mean was calculated.

We compared four groups: young and old individuals of both major and minor workers, based on lipid droplet size, the size of trophocytes and oenocytes, and the ratio of trophocytes to oenocytes. Each group contained 6 to 12 individuals, although old minor workers were excluded from analyses except for lipid droplet size for the reasons mentioned above. Data normality and homogeneity of variances were assessed prior to statistical analysis. According to the data distribution, a one-way ANOVA followed by Tukey's HSD test was applied to the size of trophocytes and oenocytes, and to the ratio of trophocytes to oenocytes. For lipid droplet size, the Kruskal-Wallis test followed by Nemenyi test was applied.

#### RESULTS

#### **Observation of foraging behavior**

A total of 784 foraging behaviors were observed. In two of the three colonies, major workers accounted for 17.0% and 11.4% of the colony workers, however none were observed outside the nest (Fig. 2). In one other colony, major workers were observed outside the nest 30 times (total n = 416). Although the proportion of major workers among foragers (7.2%) was lower than in the colony (10.6%), the difference was not statistically significant (Fisher's exact test: P = 0.23).

# Observation of defensive behavior of major workers against other ant species

Major workers plugged the nest entrance 53.7%  $\pm$  35.6 (mean  $\pm$  SD) of the time when without competitors, but the introduction of *T. congruus* increased this time in all colonies to 94.5%  $\pm$  4.4 (Fig. 3). On the other hand, the percentage of minor workers at the nest entrance did not show clear changes 44.8%  $\pm$  11.9 (no competitors) to 35.6%  $\pm$  22.8 (with competitors).

Nest entrance plugging rates were higher in the presence of competitors and when the subcaste was major workers (GLMM, Binomial, link = logit; Competitor , P < 0.001, Caste, P < 0.001; Table 1, Fig. 3). The effect of competitors on the frequency of nest entrance plugging differed between major and minor workers (Competitor \* Caste, P < 0.001; Table 1, Fig. 3). These results indicate that major workers more frequently plugged the nest entrance when other ant species were present, while minor workers did not show a similar response.

	estimate	SE	р	lower 95%CI	upper 95%CI
Intercept	-4.728	0.613	< 0.001	-5.929	-3.526
Competitor	2.561	0.244	< 0.001	2.082	3.040
Caste	4.741	0.644	< 0.001	3.479	6.003
Competitor: Caste	-2.774	2.774	< 0.001	-3.328	-2.220

**Table 1.** The effects of heterospecific competitor ant (Competitor) and subcaste (Caste) on the entrance plugging frequency. Result of Binominal GLMM (link = logit) was shown.

**Observation and quantification of fat body cells** Observations showed that young minor workers had more lipid droplets in the trophocytes than old minor workers (Fig. 4A). In contrast, trophocytes were heavily degenerated and lipid droplets were almost invisible in old minor workers (Fig. 4B). In major workers, lipid droplets were abundant in trophocytes in both young and old workers, and cell size appears to be almost unchanged (Fig. 4C, D). Quantification from images showed that lipid droplet size of trophocytes decreased significantly in old minor workers, but it did not differ between young and old major workers (Fig. 5A). Oenocyte numbers relative to trophocyte numbers did not change with age in major workers (Fig. 5B). In old minor workers, fat body tissue was severely degenerated and data could not be obtained. For both trophocytes and oenocytes, major workers had larger cell sizes than minor workers, which did not change with age (Fig. 5C, D).

# DISCUSSION

Major workers of *C. yamaokai* are rarely active outside the nest (Fig. 2) and likely specialized in plugging the nest entrance (Fig. 3). However, nest entrance plugging is low in frequency under normal conditions and becomes high in frequency when *T. congruus* were present. Unlike soldiers that actively defend outside the nest (e.g., in *Pheidole* and *Atta*; Detrain & Pasteels 1992; Powell & Clark 2004; Huang 2010), major workers of *C. yamaokai* primarily perform intranidal defense by plugging the nest entrance.

Nest entrance plugging is found in some ant species (e.g., Colobopsis and Cephalotes) where major workers are present (Powell 2008). Some of these ants have flat heads that appear to be morphologically highly specialized to nest entrance plugging. In contrast, the subgenus Myrmamblys, to which this species belongs, generally have flattened clypeus, but the head is round (Santschi 1926), suggesting relatively low morphological specialization to nest plugging. In Colobopsis, major workers are almost always observed plugging the nest entrance, even without competitors (Fujioka et al. 2019). In contrast, C. yamaokai performed plugging behavior less frequently when competitors were absent, suggesting moderate behavioral specialization to

entrance plugging. *Pheidole obtusospinosa* is an example of a major worker that, like this species, does not have a specialized head morphology for plugging and defends its nest entrance in response to the presence of competitors (Huang 2010). *Camponotus yamaokai* potentially have unicolonial characteristics, as colonies are founded by budding, and aggression between nests is low regardless of distance (Satoh 1990; Satoh & Hirota 2005). Therefore, intraspecific competition in this species is relatively small, and nest entrance defense may be primarily useful against other species, though effects of intraspecific competition should be tested in future studies.

In the fat bodies, age-dependent morphological changes in lipid droplet size, trophocyte size and oenocyte size were smaller in major workers than in minor workers (Fig. 4, 5). The trophocyte to oenocyte ratio did not change with age in major workers. Thus, physiological changes in major workers rarely occur or progress more slowly than in minor workers. Generally, in social Hymenoptera, individuals with high fat mass at younger ages engage in intranidal tasks, while those with reduced fat mass shift to extranidal tasks (Blanchard et al. 2000; Toth & Robinson 2005). In addition, lipid droplet size and fat body morphology are known to change in correlation with age in honey bees (Strachecka et al. 2017). Observations of fat body cells in this species suggest that age-dependent fat loss occurs in minor workers, suggesting that, like known monomorphic ants, their fat mass decreases and the shift to forager is correlated in minor workers. In contrast, no age-dependent decrease in fat occurred in major workers. Moreover, in this study, major workers were rarely observed performing extranidal tasks. Therefore, it is expected that agedependent task shift to foraging does not occur in major workers. This differs from what was known for workers in monomorphic ants and bees, suggesting that the stasis or slow change in the physiological state of major workers underpins the behavioral stability of major workers. However, the foraging by some major workers were observed in one colony. We speculate that very old major workers may occasionally forage. Additionally, our preliminary observations suggest that major workers live longer than minor workers (KK personal observation). Although the "old" category

in this study likely included a wide range of ages, from workers just over seven months to those near the end of their lifespan, no major workers with degenerated fat bodies were observed, unlike in minor workers. Therefore, the difference in lifespan between subcastes is unlikely to account for the observed difference in age-dependent physiological changes but this should be noted as a limitation in the current study.

In summary, this study indicates that major workers of *C. yamaokai* had specialized roles as nest entrance pluggers. Physiological changes in major workers with aging were smaller than those in minor workers. As is known for workers of ants and bees in general (Blanchard et al. 2000; Toth & Robinson 2005; Bernadou et al. 2020), minor workers lose fat and shift from nurse to forager as they age. On the other hand, physiological status of major workers may shift gradually or remain stable, reflecting the generally inactive, facultative plugging role of major workers.

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Statements and Declarations

**Competing interests** The authors declare no competing or financial interests.

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