Investigation of Age Polyethism in Food Processing of the Fungus-Growing Termite *Odontotermes formosanus* (Blattodea: Termitidae) Using a Laboratory Artificial Rearing System

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ABSTRACT Laboratory rearing systems are useful models for studying Rhinotermitid behavior. Information on the biology of fungus-growing termites, however, is limited because of the difficulty of rearing colonies in the laboratory settings. The physical structure of termite nests makes it impossible to photograph or to observe colonies in the field. In this study, an artificial rearing system for field-collected colonies of the fungus-growing termite *Odontotermes formosanus* (Shiraki) was developed to facilitate observation in the laboratory. We recorded colony activity within the artificial rearing system and documented a variety of social behaviors that occurred throughout the food processing of the colony. This complex miniature ecosystem was cooperatively organized via division of labor in the foraging and processing of plant materials, and the observed patterns largely resembled the caste and age-based principles present in *Macrotermes* colonies. This work extends our insights into polyethism in the subfamily Macrotermitinae.

KEY WORDS termite rearing, social behavior, division of labor, food foraging and processing strategy

Introduction

The development of devices to keep termite alive, observable, and detectable in laboratory has been shown to effectively enhance the study of the biology and ecology of termite. Some of these devices used against subterranean termites (Rhinotermitidae) in the laboratory have been well established (Chouvenc et al. 2011). Laboratory evaluation of termiticides (Su 2005), feeding preference (Castillo et al. 2012), and colony population size (Su 2013), etc. using the artificial termite rearing system have improved the understanding of the fundamental issues such as foraging strategy, trophallaxis, and disease resistance. More importantly, these investigations were also helpful in enhancing termite control strategy (Chouvenc et al. 2011).

Fungus-growing termites in the subfamily Macrotermitinae cultivate Basidiomycetes fungus (*Termitomyces* spp.) in their nests and have wide distribution in Africa and Asia. To date, more than half of the described Macrotermitini species belong to the genus *Odontotermes* (Darlington et al. 2008). In China, *Odontotermes formosanus* (Shiraki) (Termitidae: Macrotermitinae) is considered the most destructive pest affecting woods and dykes (Huang et al. 2001). Presently, the primary control method against *O. formosanus* is through the use of toxic bait (Huang et al. 2006). However, compared with the lower termites, the effect of toxic baits on higher termites such as *O. formosanus* is limited, and the termiticides that could eliminate or kill the entire colony of *O. formosanus* is still arguable (Ngee et al. 2004). For example, chitin synthesis inhibitors fail to work against *O. formosanus*. Moreover, the lack of a good attractant for these termites and poor delivery system of the toxicant were major obstacles towards implementing the baiting strategies against *O. formosanus* (Kasseney et al. 2011).

Termites from the subfamily Macrotermitinae demonstrate extremely complex polyethism, and detailed information of this polyethism is a prerequisite to further the understanding of many important behaviors exhibited by these termites. To date, caste- and age-related polyethism linked to food processing have been investigated in only two *Macrotermes* spp., namely, *Macrotermes subhyalinus* (Rambur) (Badertscher et al. 1983) and *Macrotermes bellicosus* (Smeathman) (Gerber et al. 1988, Leuthold et al. 1989,

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Hinze et al. 2002). Results showed that in *Macrotermes*, the older adult workers forage and consume mature fungus comb, whereas young adult workers ingest the plant materials brought in by their older nestmates as well as fungus nodules, and excrete their feces as a substrate to build up the fungal comb. In addition, the majority of foragers were major workers, whereas minor workers predominated in the queen cell. However, only one worker caste was observed in each *O. formosanus* colony, which differs from the two worker castes (major and minor) observed in *Macrotermes* (Huang et al. 2000). Thus, a fundamental study on age-related polyethism in *O. formosanus* is required to better understand how these termites feed and forage.

Macrotermitini behaviors are difficult to observe, sample, and analyze because of the challenge of developing and maintaining laboratory colonies. Past attempts to rear laboratory colonies of Macrotermitini, including species of *Macrotermes* (Sieber 1983), *Odontotermes* (Sieber 1983), and *Pseudacanthotermes* (Connetable et al. 2012), began with alate reproductive pairs, and took several years for colonies to develop. More importantly, all these colonies were located in soil, and direct observations to document termite behaviors were impossible (Huang et al. 2008, Connetable et al. 2012).

In the present study, we described an artificial rearing system in which colonies of *O. formosanus* were maintained and developed in the laboratory. The system was populated with field colonies, instead of using incipient colonies (Connetable et al. 2012). The system allowed direct observation of the colonies, so we were able to investigate the caste and age polyethism of *O. formosanus.* Results of this present study provide some insight into the nutritive cycle of *O. formosanus* colonies and how the colony system was cooperatively organized.

Materials and Methods

Collection of Termite Colonies. Five colonies of *O. formosanus* (Of312, Of313, Of315, Of316, and Of317) harboring the king and queen were collected in March 2011 from a forest area near Xiangshan (30° 14″ N, 120° 21″ E, altitude 66 m) located at the Xianghu Tourist Holiday Resort, Hangzhou City, Zhejiang Province, P.R., China. The fungus combs were ∼3 cm in height and 6 cm in diameter at the base. To prevent deaths of fungus comb caused by pathogens and water loss, all colonies were wrapped with by plastic film and transported to the laboratory within 6 h after excavation.

Design of the Artificial Rearing System and Rearing Conditions. All colonies with the presence of the royal pair were placed in the main chamber (45 by 45 by 30 cm) containing clay soil obtained from the area where the field colonies were collected, respectively (Fig. 1). As the colonies matured, a fungus comb observation chamber (45 by 45 by 30 cm) and a feeding chamber (45 by 45 by 30 cm) were connected to the main chamber by using the Plexiglas tube (66 cm in length and 2.5 cm in diameter) in March 2012. To facilitate observations and video recording, all of the main chambers were made of glass. The top of each chamber was covered with 8-cm-diameter mesh to allow air exchange between the nest and the outside environment. In addition, four 8-cm-diameter glass lids were used to cover the holes on the top of the fungus comb observation chamber (which could be replaced by a clean one once it has been plastered by termites), through which termites could be easily observed. A porous clay pot covered with a glass lid and equipped with a Plexiglas tube for adding water from the top was used to provision the termites with water and to maintain the humidity in the chamber.

To monitor termite foraging and waste management behaviors, a plane arena constructed from two sheets of transparent Plexiglas (24 cm in length by 24 cm in width by 6 mm in thickness) and sandwiched by four black Plexiglas laminates (three of which were 24 cm in length, 2 cm in width, and 2 mm in thickness, and one of which was 20 cm in length, 7 cm in width, and 2 mm in thickness) as described by Su and his co-workers (Su 2005, Chouvenc et al. 2011) was used as foraging observation arena and fecal deposition observation arena, respectively. The coiled plastic tubes (5 m in length and 5.5 mm in diameter) were used to connect the chambers to a foraging observation arena and a fecal deposition observation arena. A food container constructed from Plexiglas cup with lid was placed on the foraging observation arena. The termites were fed with partially decayed lignocellulose plant materials, including bamboo, chestnut, and pantropical weeds such as *Conyza canadensis* (L.) Cronquist (also named horse weed), and *Imperata cylindrical* (L.) P. Beauvois (also named blady grass) etc. The rearing systems were maintained in complete darkness at 27 ± 1°C and 85% relative humidity. All colonies were used for experiments since August 2012.

Imaging Setup for Analysis of Social Behavior. To facilitate observation, a camcorder (Sony DCR-HC38, Sony, Tokyo, Japan) was fixed on a tripod and placed over the glass lids of the chambers (Fig. 1), as well as transparent Plexiglas of foraging observation arena and fecal deposition observation arena to record termite behaviors. The camcorder was connected to a personal computer, on which the video-acquisition software (Scenalyzerlive 4.0, Wien, Austria) was used to analyze the termite behaviors.

Measurements of Age-Related Polyethism in Food Processing. The Macrotermitini shows sequential stages in food processing, which can be clearly divided into three tasks involved in behaviors of foraging, fungus comb building, and final feces deposition (Aanen 2006). According to this principle, four colonies (Of312, Of313, Of315, and Of317) were individually investigated using our imaging setup, the number of individuals in young and old workers performing each task were counted, that can contribute to further study of age-related labor division in food processing. The age of the worker termites was differentiated by the colors of their cuticles (Hinze et al. 2002), and the workers were classed as young workers and old workers if their abdomens were reddish brown and dark brown,

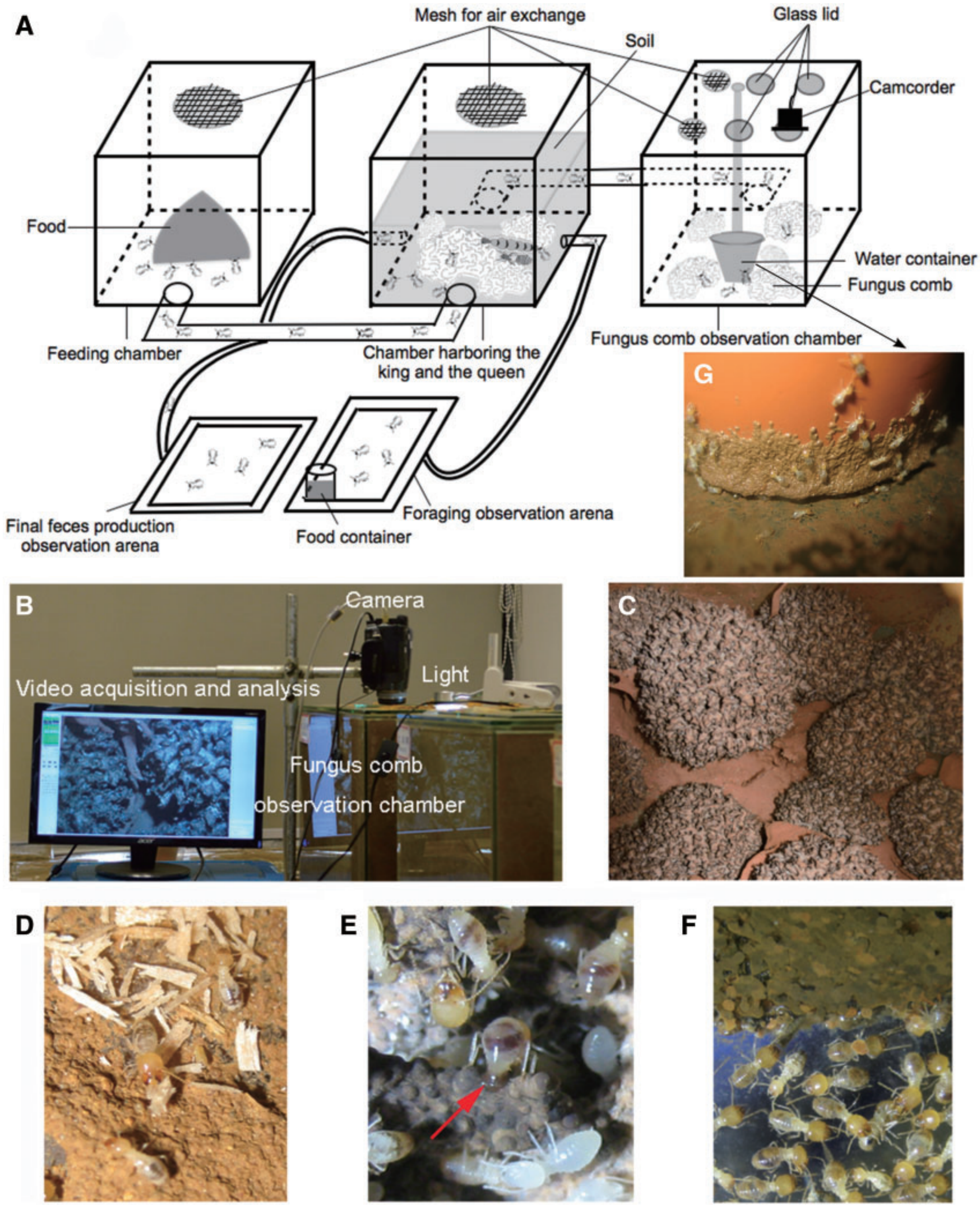

Fig. 1. Artificial rearing system for laboratory rearing and observing of the *O. formosanus* colony. (A) Schematic of the artificial rearing system. (B) Imaging setup for observing social behaviors. (C) Fungus combs without soil cover in the fungus comb observation chamber. (D) Worker termites foraging within the foraging observation arena. (E) Worker termites building a new fungus comb. (F) Worker termites producing final feces within the final feces production observation arena. (G) Worker termites drinking water from the porous clay pot.

respectively. To facilitate foraging task observation, the camcorder was placed over the entrance and exit of foraging observation arena, and the number of individuals in each age class transporting forage to nest was counted. To analyze workers engaged in fungus comb building, an arena of 6 cm in length by 4.5 cm in width over the fungus comb observation arena was randomly selected as the field of view for video recording, and the number of individuals in each age class depositing their feces on the fungus comb was counted. To measure the final feces deposition, a coiled plastic tube (3 m in length and 6 mm in diameter) was used to allow workers to deposit their feces to the fecal pile within tube, and the number of individuals in each age class depositing their feces within fecal tube was counted. Lastly, to take the activity rhythms into consideration (Hinze and Leuthold 1999), each task of individual colony was video recorded continuously for 3 h in the evening per day for 8 d in July 2013.

To determine that the role of workers in each age class during food processing, the gut contents of both young and old adult workers were randomly collected from the observation arena to be examined. The size of the wood particles and density of asexual spores of *Termitomyces* spp. in the gut contents were measured. The size of wood particles may reflect the workers' food sources, whereas the density of asexual spores reflects the task of inoculation for new fungus comb (Hinze et al. 2002). To determine this, the wood particles in freshly prepared samples of fore- or midgut of young workers, hindgut of young workers, new fungus comb, old fungus comb, fore- or midgut of old workers, and hindgut of old workers were stained with phloroglucinol–HCl solution. The diameters of the long and short axes of wood particles were measured by using a digital microscope (VHX-2000; KEYENCE, Osaka, Japan) according to Tokuda et al. (2012). The number of asexual spores in the hindgut paunch of young and old workers was determined by dyeing the hindgut paunch contents with lactophenol blue and using a hemocytometer to enumerate the spores. The volume of the hindgut paunch was estimated using a digital microscope to measure its geometrical shape as previously described (Li et al. 2012). The size of wood particles was analyzed by one-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparisons tests at $\alpha = 0.05$. The software R version 3.0.1 (www.r-project.org) was used to perform all data analyses.

Table 1. The amount of food consumed by *O. formosanus* colonies

Rearing time		Colony			
Year	Month	Of312	Of313	Of315	Of317
2012–2013	Dec.–Feb.	1,473	1,739	1,190	1,827
	Mar.–May	3,317	3,815	3,017.5	3,692
	June–Aug.	2,165	3,163	2,374	2,737.5
	Sept.–Nov.	3,548.5	4,101	3,273	3,983
	Dec.–Feb.	8,250	8,179	7,885.5	8,412.5
	Mar.–May	17,955	18,473.5	16,962	19,770
	June–Aug.	11,595	14,742	9,837	13,274

All weights in grams.

Results and Discussion

Colony Condition and Social Behavior Observation. In our study, four of the five colonies (Of312, Of313, Of315, and Of317) were successfully reared for more than 2.5 yr using the artificial rearing system. Colony Of316 died after being transferred to the laboratory and reared for 6 mo. The remaining four colonies remained in good condition during the entire rearing period as shown by following facts: The termites showed high levels of foraging activity, fungus comb building, and feces production (Fig. 1). The food consumption increased in all colonies, and the amounts consumed during 3 mo periods are shown in Table 1. Also, all colonies produced nymphs, which were usually eaten by workers before they could molt into the alate stage. Therefore, our results indicated that this glass rearing system allowed us to artificially rear *O. formosanus* colonies collected directly from the field instead of starting from primary reproductive pairs,

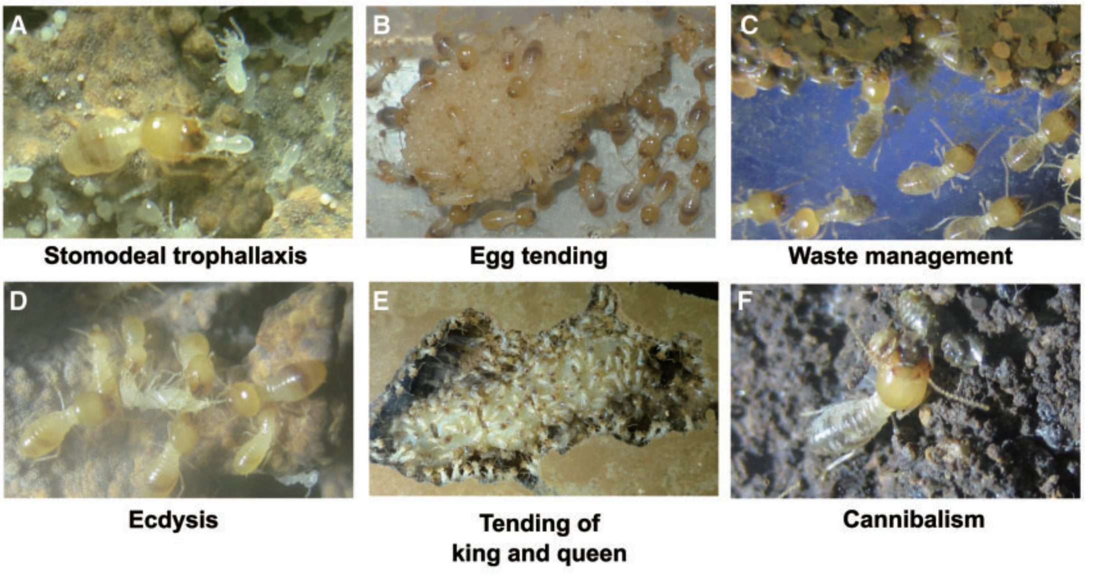

Fig. 2. Social behaviors of *O. formosanus* observed with the imaging setup. (A) Stomodeal trophallaxis between young adult worker and larvae. (B) Egg tending by young adult worker. (C) Waste management by old adult worker. (D) Ecdysis of larvae. (E) Tending of king and queen. (F) Cannibalism.

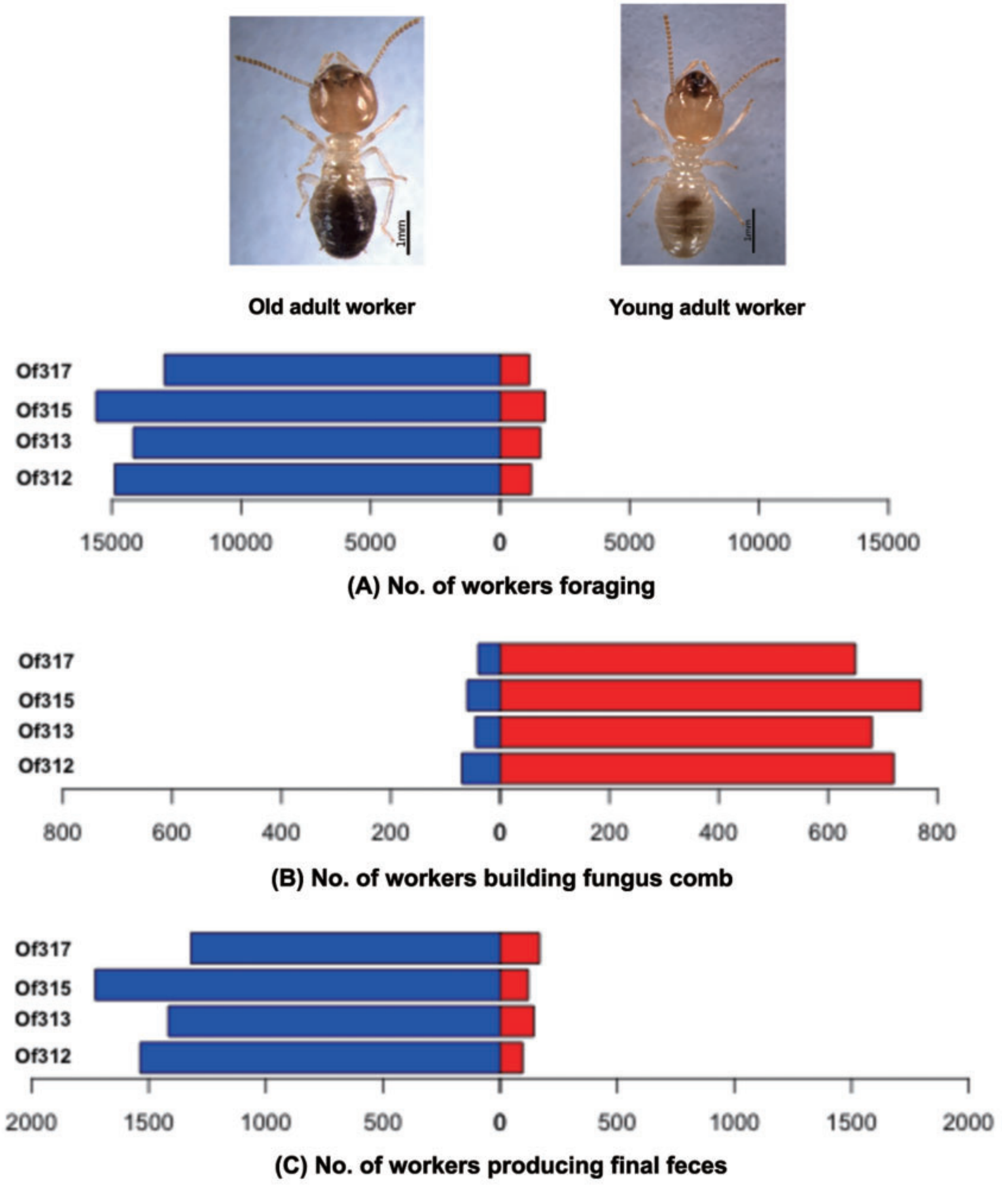

Fig. 3. Age-related division of labor in food processing. The left bars indicate the numbers of old adult workers, and the right bars indicate the numbers of young adult workers. Each bar shows data from an individual colony. (A) The number of workers foraging for food. (B) The number of workers building the fungus comb. (C) The number of workers producing final feces.

which meant that we could subsequently observe, quantify, and analyze various social behaviors in the colony soon after the colony became stable in the laboratory.

Another important feature of our rearing system is that the porous clay pot succeeded in providing the termites with water and maintaining the high humidity for the chamber (Fig. 1G), which resulted in exposed fungus comb without soil material covered by *O. formosanus* (Fig. 1C). Consequently, our imaging setup is able to observe and record the social behaviors in the respective observation arena of *O. formosanus* colony (Fig. 2).

From the fungus comb observation chamber (Fig. 2A and B), two nursing behaviors were detected. One is stomodeal trophallaxis, where larvae are fed by workers through the mouth. The other one is ecdysis, where the old cuticle of larvae is removed by workers. From the main chamber (Fig. 2C and D), tending behaviors including egg tending, as well as king and queen tending were detected. In addition, from the fecal deposition observation arena (Fig. 2E and F), waste management behaviors including feces deposition and cannibalism of sick or injured nestmates were also detected.

More importantly, the behaviors observed in our study have been showed that this system enabled us to rear exposed laboratory colonies within glass chambers instead of having them buried in the soil. Also, because the rearing system was designed to restrict functional behaviors of termites to individual arenas instead of having them occur all together, as was the case in previous field studies of *Macrotermes* colonies (Badertscher et al. 1983, Gerber et al. 1988, Leuthold et al. 1989, Hinze and Leuthold 1999), it allowed us to study individual behaviors and the complex social interactions among individuals within large groups. Thus, we were able to observe and collect detailed information about within-comb activities conveniently and accurately while minimizing interferences or disturbances.

Age-Related Polyethism in Food Processing. All colonies showed a clear age-related division of labor among foraging activity, fungus comb construction, and final feces production (Fig. 3). The older adult workers were the predominant foragers, probably because their strong mandible could cut larger wood particles into small pieces that could be carried back to nest (Supp Video 1 [online only]). The majority of workers tending on the fungus comb were young adult workers, and they wiggled their abdomen strongly until feces were deposited on the surface of the fungus comb (Supp Video 2 [online only]). However, almost all final feces producers were old workers, and they usually deposited feces into one place far from the main nest and covered them with soil. Thus, final feces production differed from that of primary feces production (also named fungus comb building (Supp Video 3 [online only]).

The hindgut paunch volumes of young and old worker termites were determined to be 0.68 ± 0.04 and 0.70 ± 0.05 µl, respectively (Table 2). The number of asexual spores within the hindgut paunch of young workers was significantly higher than that in older workers ($P < 0.05$, ANOVA). The size of wood particles significantly decreased along the digestive system ($P < 0.05$, ANOVA; Fig. 4). The wood particles in the hindgut of young workers were significantly smaller than those in their foregut and midgut ($P < 0.05$), whereas no significant change in the size of wood particles in the fungus comb was detected ($P > 0.05$). The size of wood particles also declined during passage from the foregut to the midgut to the hindgut of old workers ($P < 0.05$).

Division of labor is one of the basic traits responsible for the ecological success of social insects. In colonies of Macrotermitini, complex polyethism is important for effective plant matter processing (Badertscher et al. 1983, Gerber et al. 1988, Leuthold et al. 1989, Hinze and Leuthold 1999). However, different taxa have distinct characteristics, including habitat, diet, mound architecture, and worker morphism. For example, *O. formosanus*, like many members of the genus, builds typical subterranean nests, feeds on wood/grass/leaf litter, and forms monomorphic worker castes, whereas species of *Macrotermes* build mounds above ground, feed on grass or leaf litter, and form dimorphic worker castes (Hyodo et al. 2003, Korb 2011). These intrinsic differences make investigation of food polyethism in *Odontotermes* valuable and necessary for a better understanding of this genus.

Table 2. The number of asexual spores within the hindgut paunch of young and old adult workers of *O. formosanus*

Age	Hindgut paunch volume (µl)[a]	No. of asexual spores[b]
Young adult worker	0.68 ± 0.04	$7{,}565 \pm 789$
Old adult worker	0.70 ± 0.05	45 ± 22

[a] Hindgut paunch volume was calculated based on 15 termites for each age.
[b] Number of asexual spores was counted based on 30 termites for each age.

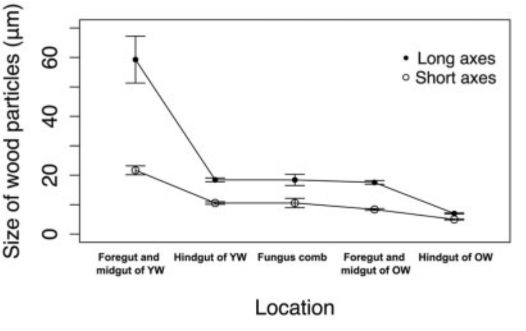

Fig. 4. Changes in size of wood particles within the digestive system of fungus-growing termites. Both long and short axes of the wood particles are shown. Each point indicates the mean ± SE of 30 measurements. YW, young adult worker; OW, old adult worker.

Using the glass system developed to artificially rear laboratory colonies of *O. formosanus*, we successfully investigated polyethism in this species in relation to fungi culture. A clear age-related behavior was found in the lignocellulosic food processing system of *O. formosanus*. Our video data showed that old adult workers were mainly responsible for the foraging task. The proportion of young adult workers involved in building the fungus comb was significantly higher than old adult workers in all observation colonies. In addition, almost all workers that deposited their feces in the final feces production observation arena were old workers. This labor division in food processing largely resembles that reported in *Macrotermes* (Badertscher et al. 1983).

The high plant materials consumption of fungus-growing termites may mainly depend on their polyethism in food supply and processing. Previous studies in *Macrotermes* found that young workers intake plant materials along with *Termitomyces* of asexual spores as a way of inoculating the new fungus comb (Hinze et al. 2002). In the present study, large amount of asexual spores and the biggest wood particles were only observed in the gut of young workers in *O. formosanus*. These revealed that, similar with the *Macrotermes*, in *Odontotermes*, it is also young workers that are responsible for the inoculation task and building the fungus comb. The smallest wood particles were found in old workers' gut confirmed that this worker group feed on fungus comb.

Results of this study, which applied our artificial rearing system in the laboratory to study the age-related polyethism in food processing of *O. formosanus*, showed that the laboratory visible rearing system was reliable in social behavior studies. Moreover, the combination results of behavioral video data and gut content determination make the resolved principles applied by the *Macrotermes* largely expandable for the *Odontotermes*, if not universal for all Macrotermitinae. In the current study, the difficulty in using bait technology against the termite pest species in the subfamilies Macrotermitinae are in large part responsible for the elaborate labor division in food processing, but the symbiotic white rot fungi *Termitomyces* involved in food processing may also contribute to the degradation of toxic bait (Lee et al. 2014). Therefore, this age-specific polyethism study was helpful in understanding of current ineffectiveness of toxic baits against Macrotermitinae, and in improving the control measure against this termite through baits strategies.

Our artificial rearing system allowed us to observe the social organization and division of labor in artificially reared colonies of *O. formosanus*. This system could be applied to other fungus-growing termites for fundamental social studies of colony foundation, caste differentiation, worker behavioral ontogeny, and plasticity of task performance, and potentially shed further light to enhance the fungus-growing termite control technology.

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