Methods for collecting large numbers of exuviae from *Coptotermes* (Blattodea: Rhinotermitidae) termite colonies

Reina L. Tong[1,], Sang-Bin Lee[1], Jayshree S. Patel[1], Thomas Chouvenc[1], and Nan-Yao Su[1]*

Abstract

The nutritional properties of subterranean termite exuviae (shed exoskeletons) are not well-known because obtaining the large quantities necessary for investigation is difficult. A method for collecting large numbers of exuviae is reported here for the Asian subterranean termite, *Coptotermes gestroi* (Wasmann) (Blattodea: Rhinotermitidae), an invasive and economically important tropical termite species. In this study, groups of 1,000 *C. gestroi* workers from 4-yr-old laboratory colonies (*n* = 3) were allowed to feed on a media pad dyed with Nile Blue A for 2 d. Approximately 16% of the original 1,000 workers did not uptake dye. These individuals were then placed into a Petri dish with dyed filter paper and checked hourly (10:00 A.M. to 10:00 P.M.) for 7 d. Newly molted workers and those individuals that started turning blue were removed to prevent feeding on exuviae. An average of 14 workers molted per d that yielded an average of 12 exuviae with an overall mean of 86 exuviae collected over the 7 d study period. We also found the number of individuals that acquired dye during the study significantly decreased by d. However, variables such as the number of exuviae, newly molted individuals, and cadavers were not correlated with d of collection because termites molt asynchronously.

Key Words: *Coptotermes gestroi*; ecdysis; exoskeleton; molting; dye; histological stains

Resumen

Las propiedades nutricionales de las exuviae de termitas subterráneas (exoesqueletos desprendidos) no son bien conocidas porque es difícil de obtener las grandes cantidades necesarias para su investigación. Aquí se informa un método para recolectar grandes cantidades de exuvias para la termita subterránea asiática, *Coptotermes gestroi* (Wasmann) (Blattodea: Rhinotermitidae), una especie de termita tropical invasora y económicamente importante. En este estudio, se permitió que grupos de 1,000 trabajadores de *C. gestroi* de colonias de laboratorio de 4 años de edad (*n* = 3) se alimentaran de una almohadilla de medio teñida con Azul Nilo A durante 2 días. Aproximadamente el 16% de los 1,000 trabajadores originales no tomaron tinte. A continuación, estos individuos se colocaron en un plato Petri con papel de filtro teñido y se controlaron cada hora (10:00 A. M. a 10:00 P. M.) durante 7 dias. Los trabajadores recién mudados y los individuos que comenzaron a ponerse azules fueron retirados para evitar alimentarse de las exuvias. Un promedio de 14 trabajadores mudaron por día, lo que produjo un promedio de 12 exuvias con una media general de 86 exuvias recolectadas durante el período de estudio de 7 días. También encontramos que el número de individuos que adquirieron tinte durante el estudio disminuyó significativamente por dia. Sin embargo, las variables como el número de exuvias, los individuos recién mudados y los cadáveres no se correlacionaron con los dias de recolección porque las termitas mudan asincrónicamente.

Palabras Clave: *Coptotermes gestroi*; ecdisis; exoesqueleto; muda; colorante; tinciones histológicas

Termites molt several times before they reach a terminal caste, but can keep molting until they die of senescence (Chouvenc & Su 2014). Shed exoskeletons (exuviae) mainly consist of nitrogenous compounds, such as protein and chitin (e.g., in cockroaches, Kramer et al. 1991) that can facilitate nutrient recycling, especially in resource scarce environments through feeding on the post-molt exuviae (Mira 2000). Termites have inherited such nitrogen-recycling behavior from their cockroach ancestors (Nalepa 1994) by the consumption of exuviae as well as cannibalism (Raina et al. 2008; Chouvenc & Su 2012; Kakkar et al. 2016a) in order to compensate for a carbon-rich but nitrogen-poor diet (Moore 1969; Mullins & Su 2018).

It was recently found that *Coptotermes formosanus* Shiraki (Blattodea: Rhinotermitidae) workers always return to the central nest (location of the reproductives and brood) to molt. This fact led to the hypothesis that workers perform an "ecdysal commute" back to the location of the brood to molt safely and successfully with the contribution of helpers (Xing et al. 2013; Du et al. 2016). However, this behavior also may result indirectly in the centralization of the nitrogen recovery process and potentially provision the queen and brood with nitrogen recycled from exuviae (Kakkar et al. 2017). In order to explore this hypothesis, hundreds of exuviae are needed to conduct experiments to quantify the nitrogen content of exuviae and its potential effect on colony growth. Collecting exuviae from *Coptotermes* termites in the field is challenging (Kakkar et al. 2016a) because termites are cryptic and the location of the central nest is not easily determined (King & Spink 1969). Even more limiting, termites have long life cycles (Kakkar et al. 2016b) and molt asynchronously (Haverty & Howard 1979; Chouvenc & Su 2014) with only approximately 1 to 2% of workers molting

[1]University of Florida, Department of Entomology and Nematology, Davie, Florida 33314, USA; E-mail: reinat@ufl.edu (R. L. T.), lsb5162@ufl.edu (S. B. L.), jayshree.patel@ufl.edu (J. S. P.), tomchouv@ufl.edu (T. C.), nysu@ufl.edu (N.-Y. S.)
*Corresponding author; E-mail: reinat@ufl.edu

per d (Raina et al. 2008; Kakkar et al. 2016b). As previously mentioned, nestmates often consume the exuviae during and immediately after the molting process (Raina et al. 2008; Xing et al. 2013; Kakkar et al. 2016a), preventing the ability to collect large quantities (> 100) of exuviae.

Termites cease feeding and clear their hindguts of symbiotic protists prior to molting (Cleveland 1925). Xing et al. (2013) described the molting cycle in termites in 5 stages: (1) intermolt, where the worker is fully functional within the colony, symbionts are present in the hindgut, and the cuticle is attached to muscles and epidermal cells; (2) pre-molt fasting, in which termites void their hindgut in preparation for molting; (3) pre-ecdysis, where the old cuticle detaches from muscles and epidermal cells, and the new cuticle begins to form; (4) ecdysis, in which the old cuticle is shed; and (5) post-ecdysis, when the new cuticle expands and hardens progressively until it reaches the next intermolt cycle (Kakkar et al. 2016a). Raina et al. (2008) determined the time of the pre-molt fast to be 6 to 10 d. Therefore, a dietary dye can be used to identify non-feeding workers, which may be in the pre-molt fasting stage, to then be separated and monitored to collect exuviae (Raina et al. 2008; Xing et al. 2013; Kakkar et al. 2016a).

The Asian subterranean termite, *Coptotermes gestroi* (Wasmann) (Blattodea: Rhinotermitidae), is among the most invasive and economically important termite species (Rust & Su 2012). *Coptotermes gestroi* is similar to *C. formosanus* in life history and behavior (Su et al. 1997), with *C. gestroi* having a tropical distribution and *C. formosanus* having a subtropical distribution (Chouvenc et al. 2016). *Coptotermes gestroi* was chosen because laboratory colonies (Chouvenc 2018) of this species consist of greater worker abundance than *C. formosanus* (Patel et al. 2020). Furthermore, *C. gestroi* has a consistent dispersal flight period in which thousands of alates (winged reproductives) may be collected (Chouvenc et al. 2017). This factor makes this species a convenient model to study nitrogen conservation in subterranean termite colonies.

During preliminary attempts to collect *C. gestroi* exuviae several issues emerged. During this process, newly molted individuals, individuals that resume feeding, and cadavers were collected. Individuals that resume feeding were the main problem for exuviae collection, as they consumed exuviae. The objective of this study was to establish a standard method to collect hundreds of exuviae from *C. gestroi* colonies and to quantify the average number of exuviae, newly molted individuals, those that resume feeding, and cadavers while observing pre-molt fasting workers. A standardized method allows for prediction of the number of workers necessary to collect in order to obtain a certain number of exuviae, and to limit stress imposed on the colonies during the collection process.

Materials and Methods

COLONY REARING

Coptotermes gestroi alates were collected from light traps in Broward County, Florida, USA, during dispersal flights in 2014 to start laboratory colonies for this study using the rearing methods of Chouvenc and Su (2017). Briefly, 1 female and 1 male were placed into a rearing unit (plastic cylindrical vial, 8 cm × 2.5 cm diam, IntraPac, Plattsburgh, New York, USA) containing 6 g of sterilized organic soil mixture (Timberline Top Soil, Oldcastle Lawn & Garden, Inc., Atlanta, Georgia, USA) and moistened with deionized water. In addition, a 3% solidified slab of agar (poured to a height of 3 cm) was added to the inner sides of 4 *Picea* sp. (Pinaceae) wood blocks (5 × 0.5 × 0.5 cm^3). This "sandwich" arrangement provided a 3 × 0.5 × 0.5 cm^3 space for access to the soil and wood in the cylinder (Fig. 1A). This chamber was then covered with a perforated lid and maintained at 28 ± 1 °C. After approximately 1 yr, each colony was moved into a larger rearing unit (plastic cylindrical vial, 6.3 cm × 4.6 cm diam, IntraPac, Plattsburgh, New York, USA) (Fig. 1B). At 2 yr, the rearing unit was uncovered and placed into a 17.5 × 12.5 × 7 cm^3 plastic container (Pioneer Plastics, Dixon, Kentucky, USA); then at 4 yr old, the colony was moved to similar containers measuring 30.5 × 45.7 × 15.2 cm^3 (Carlisle, Oklahoma City, Oklahoma, USA) (Fig. 1C, D). Moisture and wood were replenished as necessary. The central nests of the colonies were typically within the original rearing cylindrical vials (Fig. 1D).

INITIAL DYE PROCESS AND SEPARATION

One thousand *C. gestroi* workers were extracted from a vial (Fig. 1) of a 4-yr-old laboratory colony (n = 3) and placed into a glass Petri dish (90 mm diam) with a media pad (47 mm diam, AP10; Millipore SAS, Molsheim, France) dyed with approximately 1 mL of 0.05% Nile Blue A (Figs. 2, 3). The cover was placed on the Petri dish, and Parafilm ("M", Bemis; Neenah, Wisconsin, USA) was applied around the cover and bottom edge of the dish (Fig. 3). Termites were maintained in the laboratory at 28 ± 1 °C and allowed to feed on the dyed media pad for 48 h. After 48 h, non-dyed workers (potentially undergoing pre-molt fast) were counted and set aside (Fig. 2). The dyed workers were discarded.

OBSERVATION AND COLLECTION OF EXUVIAE

The non-dyed workers were placed into a new glass Petri dish (90 mm diam) with moistened filter paper (90 mm diam, 1001 090; Whatman, Buckinghamshire, United Kingdom) dyed with 0.05% Nile Blue A (Fig. 4A) in order to identify any potential workers that may resume feeding, because these workers may rapidly consume exuviae (Xing et al. 2013). To maintain relative humidity for these workers during observation, a wet media pad (47 mm diam) was taped to the inside cover of the Petri dish (Fig. 4B). The dish was then placed into a covered plastic container (17 × 12 × 6 cm^3) with the bottom lined with a paper towel dampened with water (Fig. 4E). Thereafter, Petri dishes were checked every h (10:00 A. M. to 10:00 P. M.) for molting and newly molted workers, exuviae, blue termites, and cadavers for 7 d.

Molting termites were identified by their "jackknife" position (Raina et al. 2008; Xing et al. 2013). Exuviae were removed and placed into a vial (15 mm diam × 45 mm; 1 Dram, Shell Type 1 Glass with Plug; Fisher Scientific, Pittsburgh, Pennsylvania, USA) (Fig. 4D). Workers that began to turn blue were removed to prevent them from feeding on exuviae. Newly molted workers also were removed from the Petri dish. Newly molted workers were determined either by direct observation of the molt, or by using the degree of sclerotization of the worker mouthparts as described by Kakkar et al. (2016a). Blue and newly molted workers were placed into a polystyrene Petri dish (60 mm diam) (Fig. 4C). The moisture of the Petri dishes and plastic containers was maintained as needed by adding deionized water.

STATISTICAL ANALYSIS

Mean exuviae, newly molted individuals, blue individuals (i.e., workers that acquired Nile Blue A), and cadavers collected per d from the 3 colonies were subjected to a correlation analysis to determine if time affected these variables using JMP software (JMP 15.0.0, SAS Institute, Cary, North Carolina, USA). Mean blue worker data were log transformed to meet the assumption of normality. All differences were considered significant at $\alpha < 0.05$.

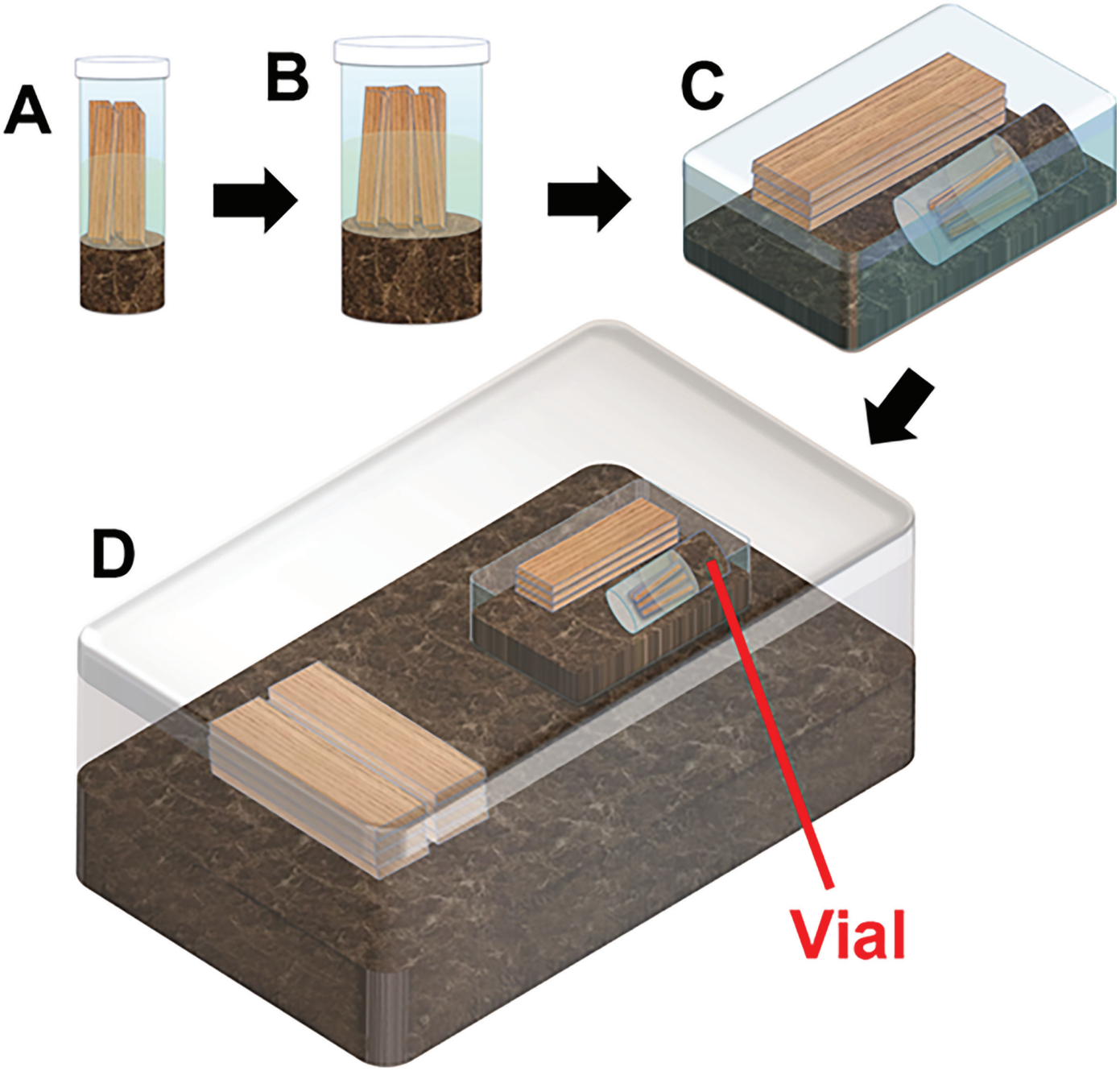


Fig. 1. Laboratory colony rearing units. (A) Plastic cylindrical vial containing moistened organic soil, wood blocks, and agar used to house termite colonies from 0 to 1-yr-old; (B) larger cylindrical vial the colony was transferred to for colonies 1 to 2-yr-old; (C) plastic container in which the uncapped cylindrical vial (pictured in B) was placed into for colonies 2 to 4-yr-old; (D) plastic container in which the uncovered container (from C) was placed into for colonies aged 4+ yr. Red line points to vial within the box that usually contains the central nest (location of reproductives and brood).

Results

After 2 d in the Petri dish with the dyed media pad, 163.0 ± 48.2 (approximately 16%) from the initial 1,000 termites did not acquire the dye and potentially were undergoing pre-molt fast. An average of 14.1 ± 5.9 workers molted per d with an average of 12.2 ± 5.5 exuviae (Fig. 5) collected during that same time period (Fig. 6). The mean number of newly molted termites over the observation period was 98.7 ± 24.2, or approximately 9.9% of the initial 1,000 termites, with approximately 1.4% of the initial 1,000 termites molting per d. A mean of 85.7 ± 19.4 exuviae were collected per 1,000 *C. gestroi* workers from the central nest over 7 d.

The d was not correlated with the number of exuviae, newly molted individuals, and cadavers collected (Fig. 6). However, the number of blue individuals collected significantly decreased by d ($r = -0.63$; $P \leq 0.01$) from the initial approximately 9% of individuals that were dyed on the first d, with most blue termites collected during the first 5 d of observation (Fig. 6C).

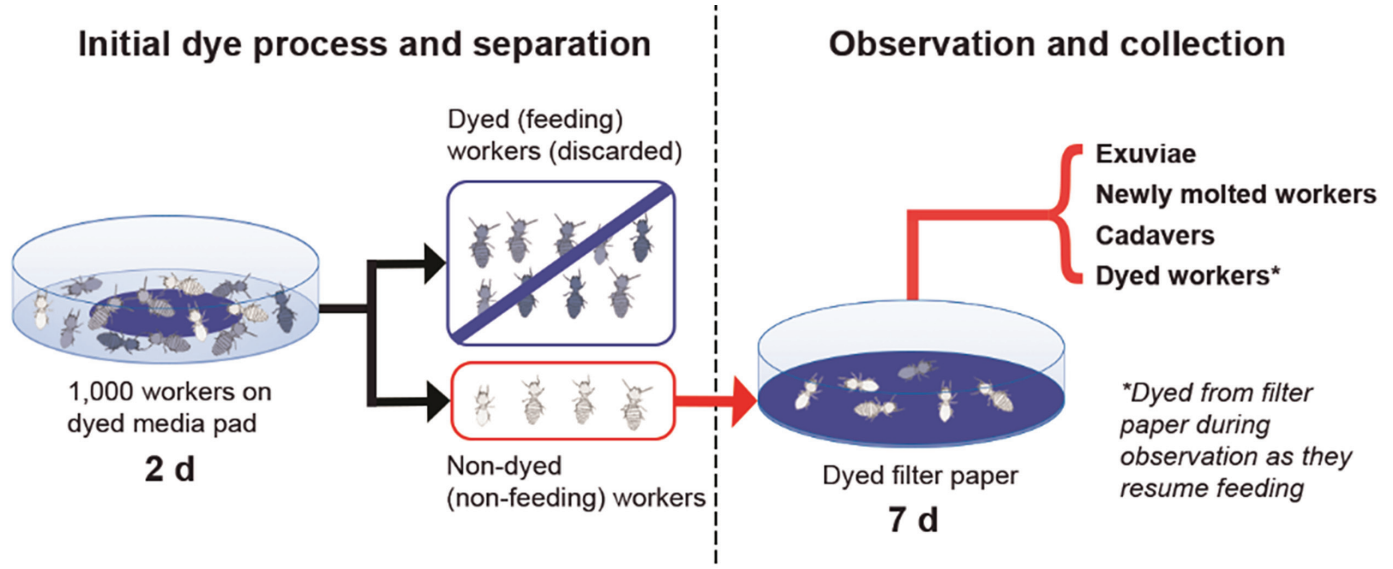


Fig. 2. Overview of the methods. *Coptotermes gestroi* workers (*n* = 1,000) were placed on a Nile Blue A-dyed media pad for 2 d in a Petri dish. Dyed and non-dyed workers were separated. Non-dyed workers, potentially under pre-molt fast, were placed on a Nile Blue A-dyed filter paper and observed for 7 d in a Petri dish. Exuviae, newly molted workers, dyed workers (that acquired dye from the filter paper during the 7 d observation), and cadavers were collected.



Fig. 3. *Coptotermes gestroi* workers (*n* = 1,000) on a media pad saturated with a 0.05% Nile Blue A and deionized water solution in a Petri dish wrapped with Parafilm.

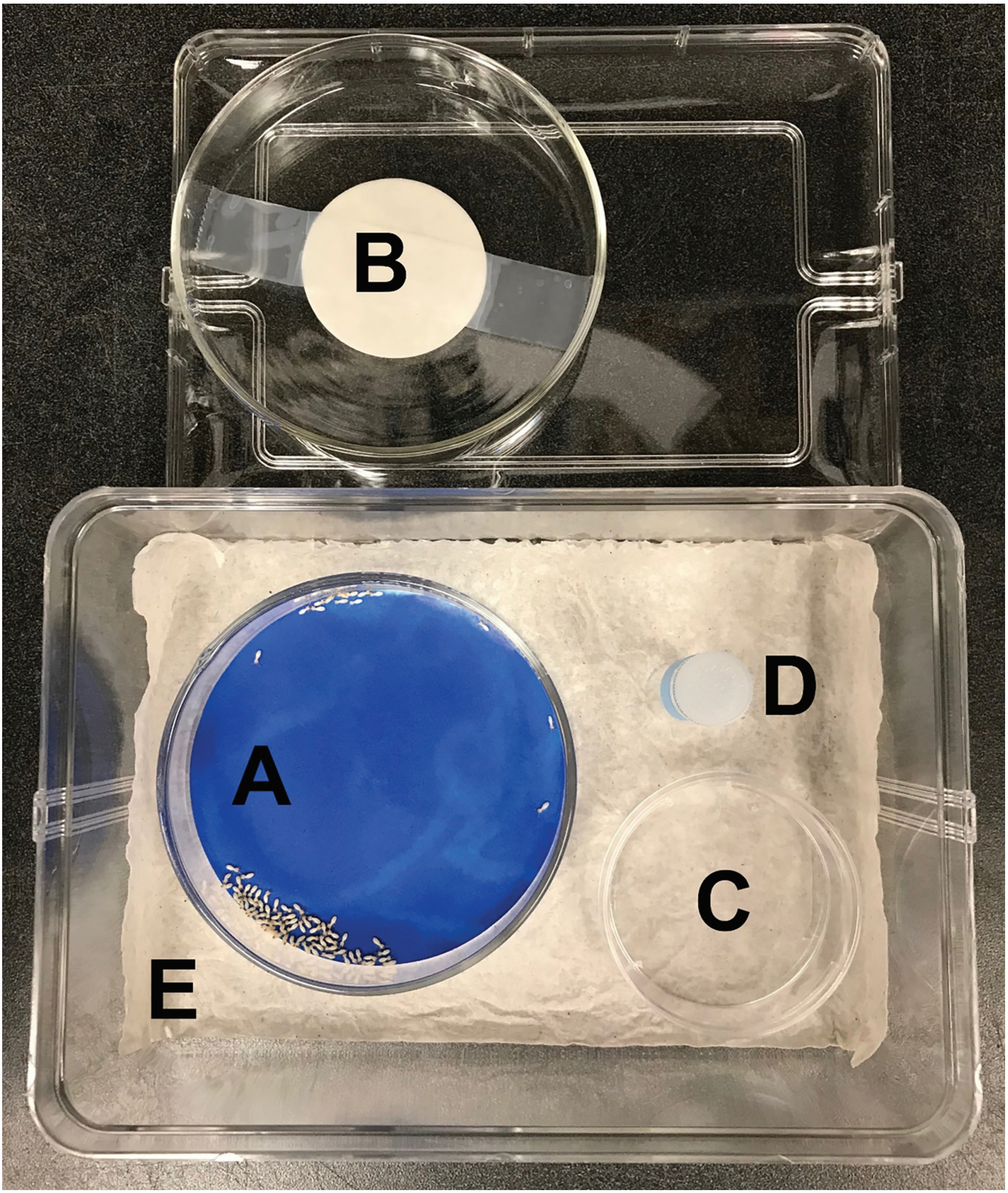


Fig. 4. (A) *Coptotermes gestroi* workers under observation on a filter paper with a 0.05% Nile Blue A and deionized water solution in a Petri dish; (B) media pad with deionized water taped to the cover of the Petri dish; (C) plastic Petri dish used to hold recently molted termites and termites that acquired dye; (D) vial used to hold exuviae; and (E) plastic container lined with a paper towel moistened with deionized water.

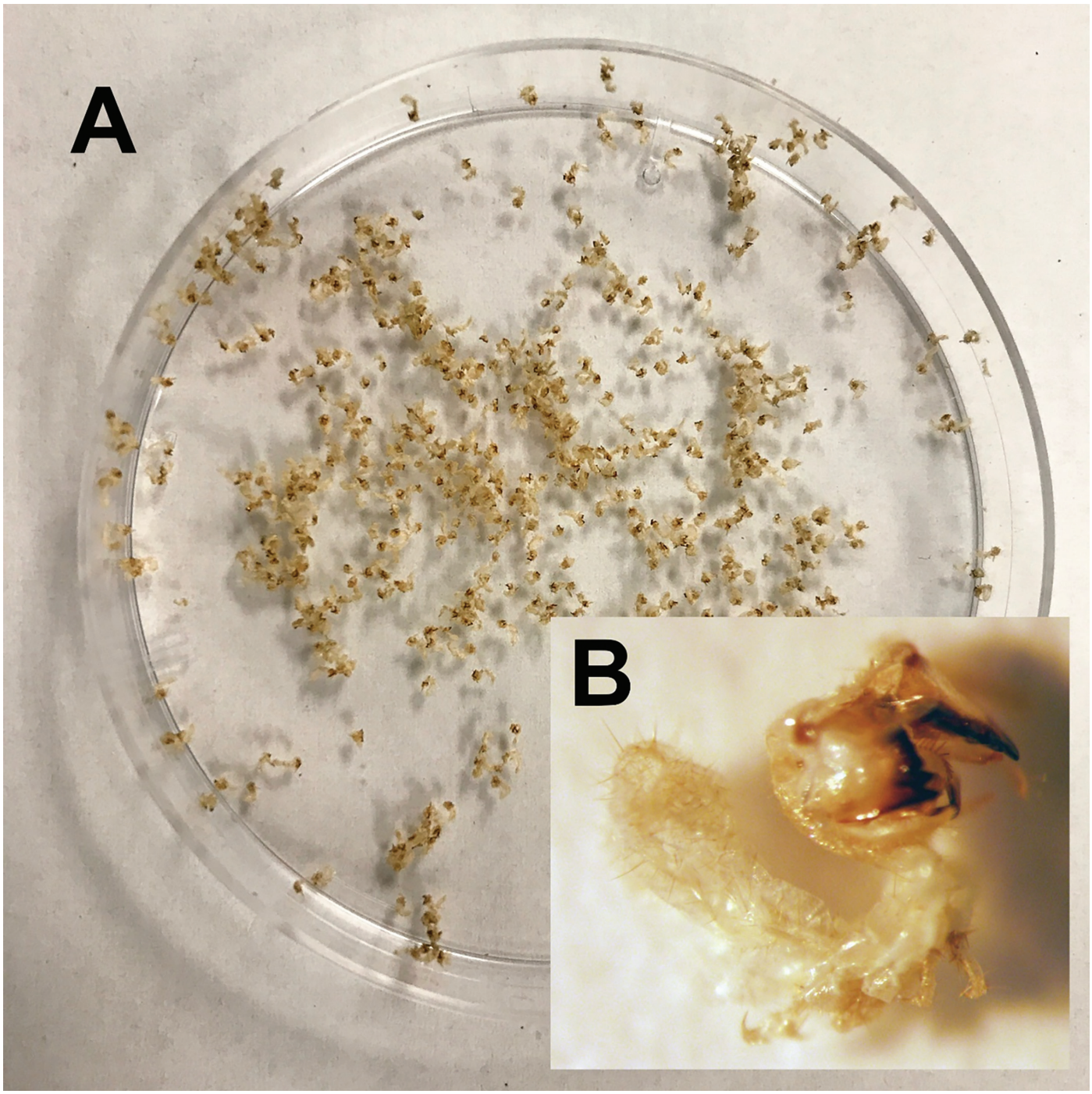


Fig. 5. (A) *Coptotermes gestroi* exuviae (approximately 550); (B) close up of exuviae.

Discussion

To the best of our knowledge, there are no publications on the collection of termite exuviae. However, this experiment takes advantage of the pre-molt fasting behavior of termite workers, allowing a dietary dye to separate individuals for monitoring of molting that was first described by Raina et al. (2008). We modified the methods previously used by Raina et al. (2008) and Xing et al. (2013) in order to facilitate the collection of large quantities of exuviae.

The duration of the initial dye administration, timing of the initial separation of dyed and non-dyed workers, moisture levels of the dish, and the number of individuals in the observation dish were all factors that affected the success of exuviae collection. The number of exuviae that were collected was lower than the number of newly molted individuals. This is because some exuviae were consumed by blue termites during the time period when observations were not performed, as inferred from the presence of partially eaten exuviae in the Petri dishes and occasional observation of blue termites feeding on exuviae.

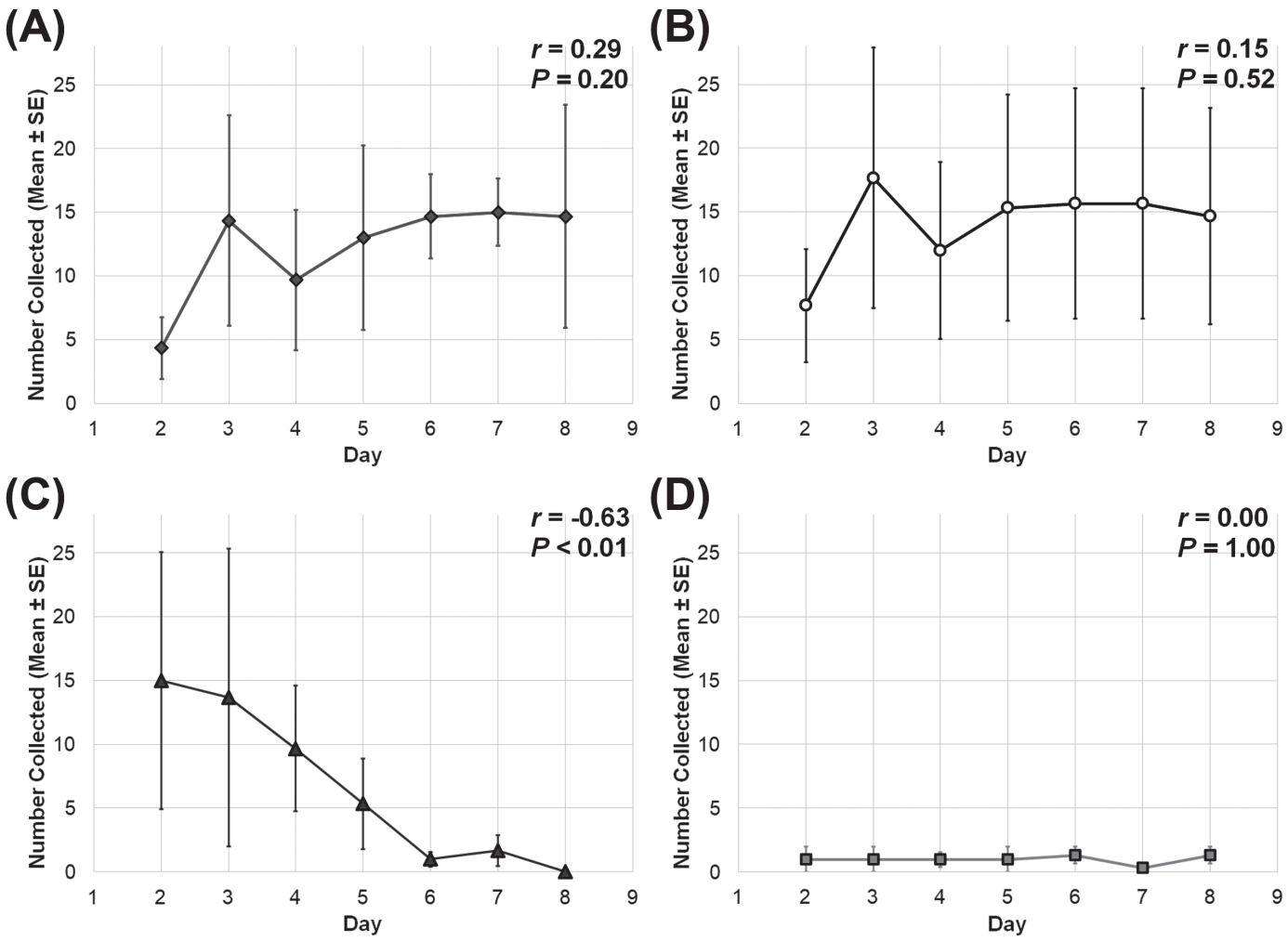


Fig. 6. Mean ± standard error of (A) exuviae collected; (B) newly molted individuals observed; (C) workers that acquired Nile Blue A dye during the 7 d observation; and (D) cadavers collected during observation of 163.0 ± 48.2 *Coptotermes gestroi* workers (*n* = 3) that were potentially in pre-molt fast.

The key step to collect exuviae, therefore, was the change from non-dyed (Raina et al. 2008; Kakkar et al. 2016a) to dyed filter paper during observation, because individuals that resumed feeding could be identified and separated, thus preventing consumption of exuviae by these non-fasting individuals. Although cockroaches feed on their own exuviae and those of their nestmates (Nalepa 1994; Mira 2000), their exuviae can be collected after molting, presumably during observation of their rearing containers (Kramer et al. 1991), and possibly due to their large size. *Periplaneta americana* (L.) (Blattodea: Blattidae) exuviae are large, weighing approximately 20 to 34 mg each (Mira 2000), compared to *C. gestroi* exuviae, which weigh approximately 0.017 mg each. In this study, non-feeding workers aided molting workers and left the exuviae in the Petri dish. When termites resumed feeding during the observation period, however, the blue dye obtained from the filter paper allowed them to be marked and removed, and decreased the incidence of the usual behavior of nestmates immediately feeding on exuviae during the molting process (Raina et al. 2008; Xing et al. 2013; Du et al. 2016).

Furthermore, the humidity conditions of the Petri dish were essential in keeping the termites alive long enough to collect the majority of their exuviae. Termites are susceptible to desiccation (Kofoid 1934), and in preliminary trials, termites did not molt successfully when conditions were too wet (e.g., when both a media pad with deionized water on the cover and Parafilm were used), and termites died when humidity was too low. In this study, deionized water was added to the filter paper, media pad, and paper towel every 2 to 3 d. It also was important to keep cadavers out of the Petri dish, because they grew opportunistic fungi (Chouvenc et al. 2012). When accounting for the number of newly molted individuals, blue individuals, and cadavers, an average of 11.0 ± 8.6 termites was not accounted for at the end of each observation and were assumed to have been cannibalized (Chouvenc 2020).

We observed a 1.4% molting ratio per d with our colonies that was similar to previous studies reported from field collected populations of *C. formosanus* (Kakkar et al. 2016b). The similar molting ratios of *C. formosanus* and *C. gestroi* may be a result of similar laboratory temperatures used during observation. Kakkar et al. (2016b) reported that workers molted at a lower rate at 21 °C (0.6% per d) than at 27 °C (2.2% per d).

Time (d) was not correlated with the number of exuviae, newly molted individuals, or cadavers collected, because termites molt asynchronously. However, the negative correlation between the number of blue termites collected and d may be due to individuals beginning to feed after the initial separation. Additionally, some individuals may not have been at the pre-molt fast stage, but were counted as non-dyed even though they contained very small amounts of dye during initial separation.

This methodology provides a means to collect hundreds of subterranean termite exuviae, a resource usually immediately consumed

by nestmates. Per 1,000 workers, approximately 80 exuviae may be collected using these methods. After an initial separation of dyed and non-dyed workers, non-dyed workers may be observed for 7 d in a Petri dish lined with moist, Nile Blue A-dyed filter paper. The dyed filter paper distinguishes feeding termites that consume exuviae and need to be separated from the observation dish. Hundreds of exuviae may be collected for future experiments studying the potential role of exuviae in nitrogen conservation strategies in termites. This method also may be applied to separate individuals by known date and time of molt, or by isolating individuals that have cleared their guts of protozoans.

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