Title: Nestmate discrimination expels intrusion of intraspecific cheaters in an ant

Authors: Takuma P. Nakamura and Shigeto Dobata

Author Affiliation: Department of General Systems Studies, Graduate School of Arts and Sciences, The University of Tokyo, Komaba, Meguro, Tokyo, 153-8902, JAPAN.

Corresponding Author: Shigeto Dobata (dobata@g.ecc.u-tokyo.ac.jp)

AUTHOR CONTRIBUTIONS

Takuma P. Nakamura: Formal analysis; software; data curation; methodology; visualization; resources; writing – original draft. Shigeto Dobata: Conceptualization; supervision; validation; software; methodology; funding acquisition; project administration; resources; writing – original draft.

ACKNOWLEDGEMENTS

We thank Kazuki Tsuji, Hiroyuki Shimoji, Tetsu Morimoto, Po-Wei Hsu, Norika Kaneda, and Haruka Asanabe for their help during the field and laboratory work. This work was partly supported by Grant-in-Aid for Scientific Research (KAKENHI; 21H02537, 21H04885, 22H02705, and 22H02364 to SD) from the Japan Society for the Promotion of Science.

DATA AVAILABILITY

All data used in this study, including movies, data table and R code, were deposited in the Dryad repository (https://doi.org/10.5061/dryad.59zw3r2h2).

Abstract

Social animals utilise various communication methods to organise their societies. In social insects, nestmate discrimination plays a crucial role in regulating colony membership. Counter to this system, socially parasitic species employ adaptive strategies to bypass their host's detection. Such relationships between hosts and social parasites could be applied to intraspecific social parasitism known in some social insects. Here we show that nestmate discrimination functions as an effective border defence against intrusion of intraspecific social parasites in the Japanese parthenogenetic ant *Pristomyrmex punctatus*. This species harbours a genetically distinct cheater lineage which infiltrates and exploits host colonies. Our behavioural observations revealed that the cheaters did not show any behavioural strategies to circumvent nestmate discrimination; most of them were eliminated through aggressions by host workers that are typically observed against non-nestmates, resulting in a very low intrusion success rate for the cheaters (6.7%). This result contrasts with the expectation from interspecific social parasitism but rather resembles the intraspecific counterpart reported in Cape honeybees (*Apis mellifera capensis*). Our study thus illustrates the role of nestmate discrimination as "social immunity" against the intrusion of cheaters, highlighting the evolutionary analogy to immune systems in multicellular organisms.

Keywords: cheating, clonal ants, eusocial insects, nestmate recognition, social cancer, social interaction

1 INTRODUCTION

Social insects, such as ants, bees, wasps and termites, employ a diverse range of communication methods to organise their societies (Wilson, 1971; 1975). Nestmate recognition, a pivotal faculty among these social insects, entails the identification of individuals either as integral members of their colony or as extraneous entities, thereby regulating the admission of individuals into the colony (van Zweden & d'Ettorre, 2010). The recognition relies on the use of body cuticular hydrocarbons (CHCs) (Lahav *et al*., 1999; Sturgis & Gordon, 2012; van Zweden & d'Ettorre, 2010). Individuals producing distinct CHC blends are identified as non-nestmates (Guerrieri *et al*., 2009; Ozaki *et al*., 2005). After nestmate recognition, agonistic defensive behaviours are directed toward non-nestmate intruders (Richard & Hunt, 2013). This mechanism, known as nestmate discrimination, acts as a colony defence by avoiding any threats to the colony posed by heterospecific and non-nestmate conspecific individuals, while also fostering preferential assistance and cooperative behaviours among closely related individuals (Crozier & Pamilo, 1996; Hölldobler & Wilson, 1990).

To bypass the chemically based nestmate discrimination, social parasites exhibit special adaptations integrating chemical and behavioural strategies, which allow them to evade host aggression, infiltrate host colonies and exploit their resources (De La Mora *et al*., 2020; Grüter *et al*., 2018; Nash & Boomsma, 2008; Rabeling, 2021). For example, in socially parasitic ants with chemical camouflage, the parasites actively perform allogrooming toward host individuals which allows them to gather chemical cues from the host body surface (Lenoir *et al*., 2001). Slave-making ants forcefully infiltrate host colonies and launch attacks toward host individuals (Kleeberg & Foitzik, 2016). All these strategies can be primarily assessed by behavioural observations as an initial step in the study of sophisticated social parasitism.

In this study, we explored the existence of an infiltration strategy in a socially parasitic "cheater" lineage of the Japanese parthenogenetic ant, *Pristomyrmex punctatus*. The cheaters exhibit genetically determined morphology distinct from their host, manifesting increased oviposition rates and abstaining from engagement in colonial tasks such as nursing and foraging (Dobata *et al*., 2009; Sasaki & Tsuji, 2003, see MATERIALS AND METHODS for details). Despite the operational nestmate discrimination system (Sanada-Morimura *et al*., 2003; Tsuji, 1988a), the singular cheater lineage permeates genetically diverse host colonies

(Dobata *et al*., 2011). This observation implies the migratory behaviour of cheaters among colonies, rendering this intraspecific phenomenon analogous to interspecific social parasitism.

Our anticipation rested on the assumption that cheaters would devise a strategy to circumvent nestmate discrimination. Considering the detrimental impact of cheater intrusion on the fitness of host individuals (Dobata & Tsuji, 2013), it is postulated that host individuals may, in turn, have evolved discriminatory behaviours specifically targeting the cheaters. Through the implementation of a colony introduction experimental design and video recording, we systematically examined whether the cheater lineage employs a specialised behavioural strategy to evade nestmate discrimination, assessed the manifestation of counter-behaviours in host individuals towards cheaters, and evaluated the success of cheater intrusion.

2 MATERIALS AND METHODS

2.1 Study species

P. punctatus is a common ant species in Japan, distinguished by the absence of the queen caste and the scarcity of males (Itow *et al*., 1984). Instead, the majority of females are morphologically workers. They have two ovarioles and engage in reproductive activities, producing offspring through thelytokous parthenogenesis (Itow *et al*., 1984; Tsuji & Dobata, 2011). *P. punctatus* follows age-polyethism, wherein young adults undertake inside-nest tasks, nurturing the brood and laying eggs, while older adults cease reproduction and participate in outside-nest tasks such as foraging (Tsuji, 1988b, 1990b). However, some individuals deviate from this age-polyethism. Previous studies have found individuals with larger body size and four ovarioles in some populations, who coexist with typical individuals in a colony (Itow *et al*., 1984; Tsuji & Dobata, 2011). These individuals are infrequently active outside the nest, refrain from foraging or nursing, but exhibit higher egg-laying rates compared to typical individuals (Sasaki & Tsuji, 2003). Among those sporadic individuals, population genetic analyses have identified a monophyletic lineage, termed the “cheater” lineage. The cheater lineage is genetically distinct from, yet nested within, lineages of typical individuals, hereafter denoted as “worker” lineages (Dobata *et al*., 2009, 2011). The above-mentioned set of individual phenotypes of the cheater lineage, likely equipped with a heritable

basis, was demonstrated to have a detrimental fitness effect on the natal colony (Dobata *et al*., 2009; Dobata & Tsuji, 2013).

2.2 Sampling

Colonies of *P. punctatus* were collected in Kihoku, Mie Prefecture, Japan, from May 18 to 20, 2022. The presence/absence of cheaters was confirmed in the field by spreading the colony on a white plastic tray and visually inspecting for 30 person-minutes. The colonies were then brought back to the laboratory, were housed in plastic trays containing nest material (soil and dead leaves) and were subjected to natural light-dark cycles at 25°C. Glass test tubes were employed as nesting sites, filled with water and covered with absorbent cotton. To prevent ant escape, the tray walls were coated with Fluon®. The colonies received insect jelly (Pro Jelly, KB Farm, Japan) twice a week. The colonies used in the experimental procedures are listed in Table S1.

2.3 Colony introductions

We conducted a “colony introduction” assay following the methodology outlined by Roulston *et al*. (2003). A set of trials consisted of the introduction of (1) cheater, (2) forager, and (3) nurse non-nestmates, all obtained from the same source colony (intruder-source colony). Intruders were individually introduced into an experimental colony (hereafter referred to as host colony) artificially made from a field colony lacking cheaters (host-source colonies). We used five host-source colonies (A, B, C, D, and F) and five intruder-source colonies (H, I, J, K, and M), resulting in 15 combinations detailed in Table 1. As a negative control, (4) a nestmate nurse from the host-source colony was introduced three times per colony. Nurses are generally expected to be less aggressive than foragers (Larsen *et al*. 2016), which is true for *P. punctatus* (T. Nakamura and S. Dobata, personal observation). The experiments were conducted from August 22 to September 11, 2022, during a later stage of brood production.

147 **Table 1**. Combination of source colonies in the colony introduction assay.

host-source / intruder-source	A	B	C	D	F
H	✓			✓	✓
I	✓	✓			✓
J	✓	✓	✓		
K		✓	✓	✓	
M			✓	✓	✓

148

149 For each combination with a tick symbol, one cheater, one forager, and one nurse were
150 introduced.

151

152 Plastic containers (120 mmL × 80 mmW × 70 mmH) with a plaster floor and
153 Fluon®-coated walls were prepared. Additionally, a shallow square hole
154 (approximately 27 mm square, 2 mm depth) was created on the floor as a nest
155 site, covered with a 30 mm square glass plate (Figure 1). A total of 150 adults,
156 including nurses and foragers, were haphazardly taken from a host-source colony
157 and were placed in a container. Most individuals became distributed in the nest
158 site within 24 hours (Figure 1) and thereby formed a host colony of this queenless
159 species. The holes in the cases were moistened by allowing water to seep
160 through the surrounding plaster.

Figure 1. An experimental host colony with an intruder (arrowhead) being introduced. Scale bar: 20 mm.

On the same day, intruders were extracted from their source colony as follows: the test tube was first removed from the tray and placed in a plastic container for an hour; then individuals outside the test tube were separated every hour as foragers, repeated for three times; and finally, individuals were forced to exit the test tube and those who carry broods (eggs, larvae, or pupae) were separated as nurses. If there were no broods in the nest, broods from another colony were temporarily given to confirm their behaviours. The separated individuals were marked on the abdomen with a paint marker (Paint Marker px-20 Pink, Mitsubishi Pencil, Tokyo, Japan) and were placed in another container with a sufficient number (ca. 1000) of individuals from their source colony until used in the introduction assays on the next day.

Approximately 24–26 hours after the host colony was established, a single painted individual was introduced onto the foraging arena of the host colony using a plastic bridge (Figure 1). The entire container was video-recorded for at least

300 seconds between 1000 and 1800 hours with a Logicool C920n HD PRO webcam and Logicool Capture software (Logicool Co., Tokyo, Japan). One hour after introduction, we checked inside the container to observe the survival and location (inside or outside of the nest) of the intruder. Individuals unable to move were tentatively treated as dead. The nest site was then moistened again as described above. After 24 hours of introduction, the same checking procedure was conducted. The intruder was then collected and stored at –80°C. Sometimes the paint was removed from the intruder after introduction, preventing identification of nurses and foragers. During experiments, we repeated the above procedure using a focal intruder type up to three times until the intruder's fate and location was confirmed after 24 hours for each combination of host-source and intruder-source colonies (Table 1). Nevertheless, these additional replicates were eventually used only for the behavioural analysis, not for the analyses of survival and location of the intruders.

2.4 Behavioural analysis

We analysed the video-recorded behaviours of the intruder and the host individuals during the colony introductions. Each video was assigned a random ID to blind the intruder types as much as possible. Behavioural scores were derived from Suarez *et al*. (1999) with slight modification: 0 = ignore (a focal individual shows no interest even though the interactant makes contact using her antennae); 1 = touch (antennal contacts); 2 = avoid (an encounter resulting in the retreat of a focal individual); 3 = aggression (a focal individual opens their mandibles to threaten or bite the interactant); and 4 = fighting (prolonged biting, often resulting in the immobilisation of the interactant). These scores were assigned for the behaviours of the intruder and a host individual, respectively, at each encounter. An encounter was recorded when at least one individual performed 1 = touch or an action with a higher score.

We recorded the behavioural scores until 25 encounters were made by an intruder, following the method of Roulston *et al*. (2003). In the case of an encounter consisting of more than one behavioural type (e.g., 1 and 3), it was represented by the behaviour with the highest score. In the case of immobilisation after fighting, another behavioural type “immobilised” was assigned. The net duration (seconds) that the intruder was able to stay in the host’s nest during the first 300 seconds after introduction were also recorded.

2.5 Statistical analyses

All statistical tests were conducted using R 4.2.2 (R Core Team, 2022) with the significance level α = 0.05. For the fate of the intruders, the data were treated as binary response variables: survival (alive = 1, dead = 0) and location (inside the nest = 1, outside the nest = 0) of intruders. For each non-nestmate type × colony combination, only the first introduction trial was considered. The data were then aggregated across pairs of source colonies into a cross tabulation table, which was analysed using Fisher's exact tests implemented in the fisher.test() function. For each test, a pair of intruder types was compared at one or 24 hours from the onset. Bonferroni correction was applied to address multiple comparisons, implemented in the fisher.multcomp() function of the “RVAideMemoire” package.

To compare behavioural compositions among intruder types, ANOSIM analysis was conducted using the Aitchison distance within the “vegan” package. Data from the additional replicates described above were included in the following analyses. The behavioural datasets of intruders and host individuals were analysed separately. The dataset comprised the ratio of each behaviour, with the exclusion of “immobilised,” and underwent a centred log-ratio (clr) transformation, following the multiplicative replacement strategy (Martín-Fernández, 2003): Instances where the ratio equalled 0 were substituted with 0.008, corresponding to 1/5 of the minimum observed ratio (= 1/25). Analysis was performed between all pairs of types and Bonferroni correction was applied for each pair to account for multiple comparisons. Statistically significant differences indicate that the two types are different in their behavioural compositions.

The effects of intruder types on behavioural features characterising each encounter were examined through the application of generalised linear mixed models (GLMMs). The datasets of intruders and host individuals were analysed separately. The aggregate count of hostile behaviours (scores ≥ 2) out of (25 – counts of “immobilised”) encounters were treated as response variables with binomial errors and logit-link. The combination of host- and intruder-source colonies was treated as a random effect. Model fitting was executed using the glmer() function from the “lme4” package. Pairwise comparisons among the various intruder types were conducted using the Tukey method, implemented through the glht() function in the “multcomp” package.

3 RESULTS

3.1 Colony introductions

All intruders survived one hour after introduction (Figure 2A). After 24 hours, all nurses, irrespective of their source colony of origin (non-nestmate or nestmate), exhibited continued survival, while non-nestmate foragers and cheaters showed lower survival rates compared to non-nestmate or nestmate nurses, and these differences were statistically significant (Figure 2B). Regarding intruder location, non-nestmate intruders were statistically significantly less likely to be found inside the nest after one hour of introduction, compared with nestmate nurses (Figure 2A), indicating lower rates of early success for non-nestmates in intruding into the host's nest. After 24 hours, non-nestmate nurses demonstrated greater success in intruding into the host's nest than non-nestmate foragers and cheaters, which was not statistically significantly different from the intrusion success of nestmate nurses (Figure 2B). One out of 15 non-nestmate cheaters (6.7%) survived 24 hours and successfully intruded into the host's nest site.

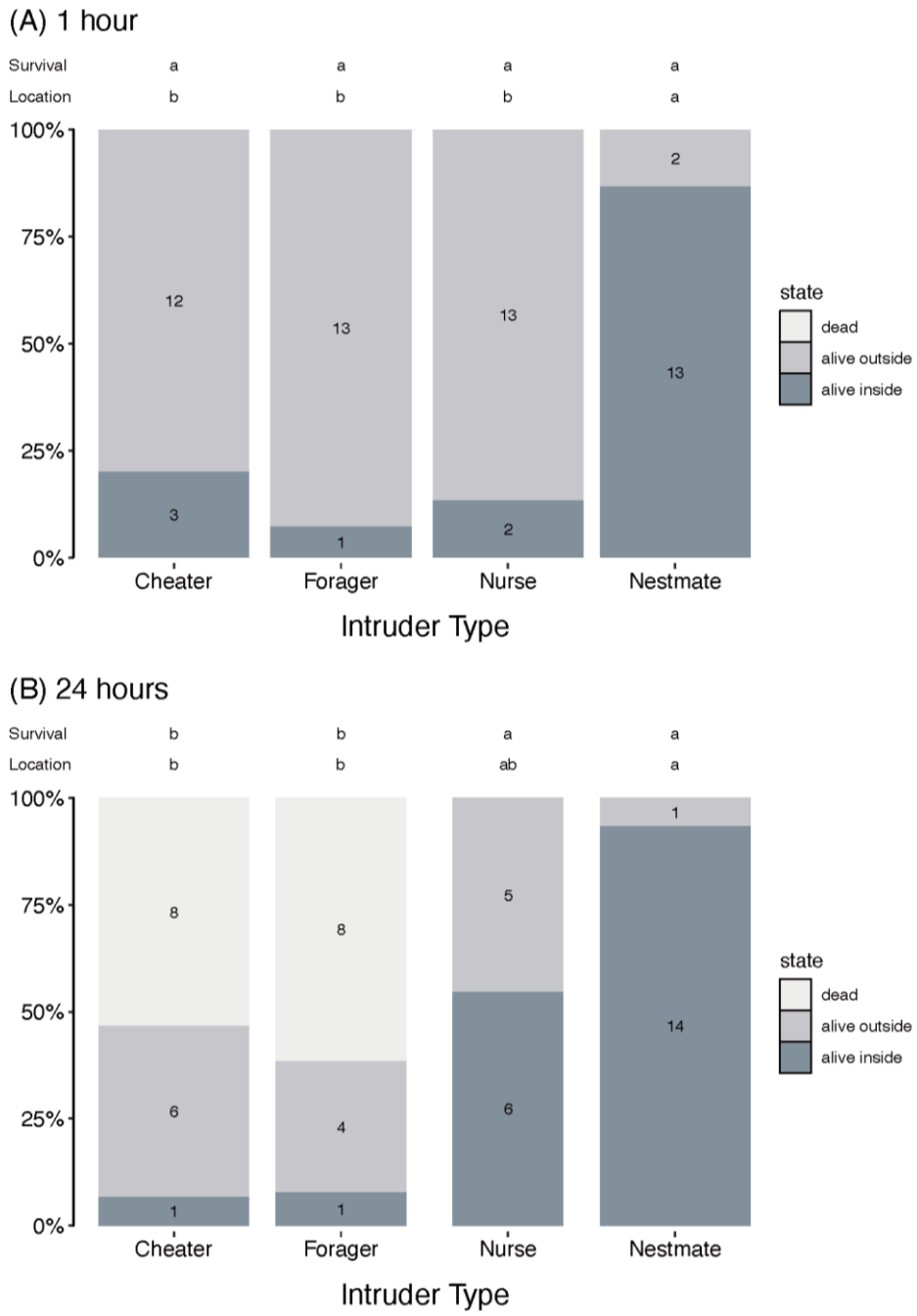

Figure 2. Results of colony introduction experiments. (A) after 1 hour and (B) after 24 hours of introduction. Bar width corresponds to the total number of trials. Different letters at the top indicate statistically significant differences among intruder types. Numbers in the bars indicate the number of individuals in each state.

3.2 Behavioural analysis

ANOSIM showed that for both intruders (Figure 3A) and host individuals (Figure 3B), there was no statistically significant difference between non-nestmate types in the composition of behavioural categories (after Bonferroni correction; intruders: $R \leq 0.119$, $p \geq 0.020$; host: $R \leq -0.004$, $p \geq 0.479$). However, a statistically significant difference was observed between any non-nestmate type and nestmate nurse (intruders: $R \geq 0.169$, $p \leq 0.005$; host: $R \geq 0.232$, $p \leq 0.003$).

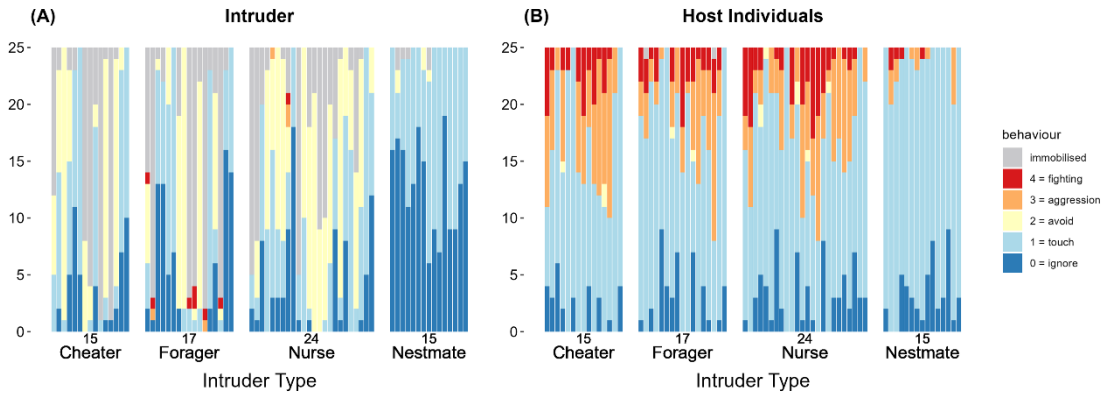

Figure 3. Frequencies of behavioural categories exhibited by (A) intruders and (B) host individuals in colony introduction experiments. Each vertical bar corresponds to an introduction trial, with their left-to-right arrangement indicating the same trial between (A) and (B).

Hostile behaviours (i.e., the composite category of 2 = avoid; 3 = aggression; 4 = fighting; note that the category “immobilised” was excluded) were more likely to be observed during intrusions by non-nestmates compared to those by nestmates for both intruders ($z \geq 3.862$, $p < 0.001$; Figure S1) and host individuals ($z \geq 3.075$, $p \leq 0.009$; Figure S2), and the difference was statistically significant (GLMM with binomial errors and logit-link, multiple comparisons with Tukey’s method).

4 DISCUSSION

In general, social parasites are expected to show adaptive traits that help them to reap benefits from their hosts. We aimed at identifying such adaptation at the initial stage of host-social parasite interactions, namely the intrusion into the host colony. In *P. punctatus*, Dobata *et al*. (2011) found individuals of the same cheater lineage in multiple host colonies with different genetic backgrounds, which suggests migration of cheaters among colonies. Therefore, our initial hypothesis presumed that cheaters would develop behavioural strategies to evade nestmate discrimination.

Nonetheless, we did not observe any distinct behaviours among the cheaters which contribute to social parasitism. ANOSIM analysis did not show statistically significant differences in the compositions of behaviours as intruders between cheaters and non-nestmate workers (Figure 3A). Focusing on hostile behaviour as intruders (i.e., avoid, aggression, and fighting), no statistically significant disparity was detected between cheaters and non-nestmate nurses (Figure S1). These results provide support against the presence of adaptive behaviours of cheaters when infiltrating other colonies.

Our analyses also suggested that behaviours of host individuals against the intruders did not differ among non-nestmates, both in the behavioural compositions and in hostile behaviours (Figures 3B and S2). Moreover, cheaters showed lowered success rates of survival and being settled within the nest after 24 hours (Figure 3B), which are typical reactions of workers in the face of non-nestmates. These findings suggest that the cheaters are treated as non-nestmates by host workers, at least in the intrusion stage. In this respect, cheaters of *P. punctatus* contrast with other socially parasitic ants that employ chemically distinctive strategies to evade detection and aggression by hosts, such as mimicry, camouflage and stealth (Akino, 2008; Lenoir *et al*., 2001; Nehring *et al*., 2015).

It can be argued that the nestmate discrimination of *P. punctatus* serves as a border defence against intrusion of the conspecific and exogenous cheaters. In general, nestmate discrimination functions as defence mechanisms against any selfishness exhibited by non-nestmates, including food-theft, brood-robbing and brood parasitism (Breed *et al*., 2012; Downs & Ratnieks, 2000), and against pathogens likely attached to non-nestmates (Lemanski *et al*., 2021). Here, an

evolutionary analogy can be drawn between nestmate discrimination of colonies and self/non-self discrimination of immune systems in multicellular organisms, with the former often being likened to “social immunity” (Cremer & Sixt, 2009; Pull & McMahon, 2020). Likewise, in *P. punctatus*, a previous study described the cheaters as transmissible “social cancers” that originated from the host itself (Dobata *et al*., 2011), which is analogous to transmissible cancers reported in some animals (Ågren *et al*., 2019; Dobata & Tsuji, 2009; Dujon *et al*., 2020). The apparently effective avoidance of cheater intrusion through nestmate discrimination illustrates the functionality of social immunity.

The apparent lack of any intrusion strategies might be explained by the relatively short evolutionary history of the cheater lineage in *P. punctatus*, which was previously estimated as 200-9200 generations (Dobata *et al*., 2011). In other words, sufficient generation time has not yet elapsed for the cheaters to adapt against the nestmate discrimination. Meanwhile, our study does not rule out the possibility of any post-intrusion strategies, behavioural or chemical, which become functional after the successful settlement of cheaters in a new host colony (Cini *et al*., 2019). A previous study found that a higher proportion of cheaters in a colony was associated with a higher tendency for a nestmate worker to be found outside their nest (i.e., to be a forager; Dobata & Tsuji, 2013). Future studies should examine whether any manipulative and counter-manipulative features can characterise the cheater-worker interactions in *P. punctatus*.

In the present experiment, the probability with which a cheater successfully settled in the host’s nest after 24 hours was 6.7% (= 1/15; Figure 3B). A similar but slightly higher probability of intrusion success (15%) was observed in the Cape honeybee, *Apis mellifera capensis* (Beekman *et al*., 2002). In this honeybee subspecies, female workers can reproduce through thelytokous parthenogenesis, invade colonies of the subspecies *A. m. scutellata*, engage in social parasitism, and ultimately dismantle the host colony (Neumann & Moritz, 2002; Yagound *et al*., 2020). However, they lack a distinct strategy during intrusion and are often rejected by the host *A. m. scutellata* workers (Neumann & Pirk, 2019).

Both in *P. punctatus* and *A. m. capensis*, the socially parasitic strains lead to the eventual collapse of the host colonies within several generations, and it seems that the observed probabilities of intrusion success might be sufficient for the social parasites to persist at least in a short term. It is worth noting that a higher probability of intrusion success would act as a “double-edged sword” by leading

358 to the pandemic-like exhaustion of the entire host population. How the observed
359 intrusion success contributes to the persistence of the social parasite lineages
360 deserves further theoretical considerations (cf. Brandt *et al*., 2005).

361 An unexpected result of our experiment was that all non-nestmate nurses
362 survived after 24 hours of experimental introduction, with over a half found inside
363 the host nest-site (Figure 3B). This result was in stark contrast with the fate of
364 non-nestmate foragers and cheaters, which showed lowered survival as
365 expected from non-nestmate discrimination. Despite encountering aggression
366 and rejection upon introduction, the non-nestmate nurses appeared to gain
367 acceptance from their host colony. In *P. punctatus*, nurses are younger adults
368 and are capable of thelytokous reproduction, whereas foragers cease
369 reproduction with degenerated ovarioles (Tsuji, 1990b). Therefore, from the kin
370 selection point of view, the apparent selective acceptance of non-nestmate
371 nurses should lower the relatedness within a host colony and thus diminish the
372 inclusive fitness of the host worker. Previous studies found a certain level of
373 flexibility in the nestmate discrimination in *P. punctatus* (Sanada-Morimura *et al*.,
374 2003; Tsuji, 1990a). Although further examination is beyond the scope of our
375 current study, future work is needed to clarify the fate of non-nestmate nurses as
376 a potential source of genetic heterogeneity observed in the field colonies (Nishide
377 *et al*., 2007; Satow *et al*., 2013).

378

REFERENCES

Ågren, J. A., Davies, N. G., & Foster, K. R. (2019). Enforcement is central to the evolution of cooperation. *Nature Ecology & Evolution*, **3**(7), 1018–1029. doi:10.1038/s41559-019-0907-1

Akino, T. (2008). Chemical strategies to deal with ants: A review of mimicry, camouflage, propaganda, and phytomimesis by ants (Hymenoptera: Formicidae) and other arthropods. *Myrmecological News*, **11**, 173–181.

Beekman, M., Wossler, T. C., Martin, S. J., & Ratnieks, F. L. W. (2002). Parasitic Cape honey bee workers (*Apis mellifera capensis*) are not given differential treatment by African guards (*A. m. scutellata*). *Insectes Sociaux*, **49**(3), 216–220. doi:10.1007/s00040-002-8304-0

Brandt, M., Foitzik, S., Fischer-Blass, B., & Heinze, J. (2005). The coevolutionary dynamics of obligate ant social parasite systems–between prudence and antagonism. *Biological Reviews*, **80**(2), 251–267. doi:10.1017/S1464793104006669

Breed, M. D., Cook, C., & Krasnec, M. O. (2012). Cleptobiosis in social Insects. *Psyche: A Journal of Entomology*, 484765. doi:10.1155/2012/484765

Cini, A., Sumner, S., & Cervo, R. (2019). Inquiline social parasites as tools to unlock the secrets of insect sociality. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **374**(1769), 20180193. doi:10.1098/rstb.2018.0193

Cremer, S., & Sixt, M. (2009). Analogies in the evolution of individual and social immunity. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **364**(1513), 129–142. doi:10.1098/rstb.2008.0166

Crozier, R. H., & Pamilo, P. (1996). *Evolution of Social Insect Colonies: Sex Allocation and Kin Selection*. Oxford University Press.

De La Mora, A., Sankovitz, M., & Purcell, J. (2020). Ants (Hymenoptera: Formicidae) as host and intruder: recent advances and future directions in the study of exploitative strategies. *Myrmecological News*, **30**, 53–71. doi:10.25849/myrmecol.news_030:053

Dobata, S., Sasaki, T., Mori, H., Hasegawa, E., Shimada, M., & Tsuji, K. (2009).

Cheater genotypes in the parthenogenetic ant *Pristomyrmex punctatus*. *Proceedings of the Royal Society B: Biological Sciences*, **276**(1656), 567–574. doi:10.1098/rspb.2008.1215

Dobata, S., Sasaki, T., Mori, H., Hasegawa, E., Shimada, M., & Tsuji, K. (2011). Persistence of the single lineage of transmissible “social cancer” in an asexual ant. *Molecular Ecology*, **20**(3), 441–455. doi:10.1111/j.1365-294X.2010.04954.x

Dobata, S., & Tsuji, K. (2009). A cheater lineage in a social insect. *Communicative & Integrative Biology*, **2**(2), 67–70. doi:10.4161/cib.7466

Dobata, S., & Tsuji, K. (2013). Public goods dilemma in asexual ant societies. *Proceedings of the National Academy of Sciences*, **110**(40), 16056–16060. doi:10.1073/pnas.1309010110

Downs, S. G., & Ratnieks, F. L. W. (2000). Adaptive shifts in honey bee (Apis mellifera L.) guarding behavior support predictions of the acceptance threshold model. *Behavioral Ecology*, **11**(3), 326–333. doi:10.1093/beheco/11.3.326

Dujon, A. M., Gatenby, R. A., Bramwell, G., MacDonald, N., Dohrmann, E., Raven, N., Schultz, A., Hamede, R., Gérard, A.-L., Giraudeau, M., Thomas, F., & Ujvari, B. (2020). Transmissible cancers in an evolutionary perspective. *iScience*, **23**(7), 101269. doi:10.1016/j.isci.2020.101269

Grüter, C., Jongepier, E., & Foitzik, S. (2018). Insect societies fight back: The evolution of defensive traits against social parasites. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **373**(1751), 20170200. doi:10.1098/rstb.2017.0200

Guerrieri, F. J., Nehring, V., Jørgensen, C. G., Nielsen, J., Galizia, C. G., & d’Ettorre, P. (2009). Ants recognize foes and not friends. *Proceedings of the Royal Society B: Biological Sciences*, **276**(1666), 2461–2468. doi:10.1098/rspb.2008.1860

Hölldobler, B., & Wilson, E. O. (1990). *The Ants*. Harvard University Press.

Itow, T., Kobayashi, K., Kubota, M., Ogata, K., Imai, H. T., & Crozier, R. H. (1984). The reproductive cycle of the queenless ant *Pristomyrmex pungens*. *Insectes Sociaux,* **31**(1), 87–102. doi:10.1007/BF02223694

Kleeberg, I., & Foitzik, S. (2016). The placid slavemaker: Avoiding detection and

conflict as an alternative, peaceful raiding strategy. *Behavioral Ecology and Sociobiology*, **70**(1), 27–39. doi:10.1007/s00265-015-2018-6

Lahav, S., Soroker, V., Hefetz, A., & Vander Meer, R. K. (1999). Direct behavioral evidence for hydrocarbons as ant recognition discriminators. *Naturwissenschaften*, **86**(5), 246–249. doi:10.1007/s001140050609

Larsen, J., Nehring, V., d'Ettorre, P., & Bos, N. (2016). Task specialization influences nestmate recognition ability in ants. *Behavioral Ecology and Sociobiology*, **70**, 1433–1440. doi:10.1007/s00265-016-2152-9

Lemanski, N., Silk, M., Fefferman, N., & Udiani, O. (2021). How territoriality reduces disease transmission among social insect colonies. *Behavioral Ecology and Sociobiology*, **75**(12), 164. doi:10.1007/s00265-021-03095-0

Lenoir, A., D'Ettorre, P., Errard, C., & Hefetz, A. (2001). Chemical ecology and social parasitism in ants. *Annual Review of Entomology*, **46**, 573–599. doi:10.1146/annurev.ento.46.1.573

Martín-Fernández, J. A. (2003). Dealing with zeros and missing values in compositional data sets using nonparametric imputation. *Mathematical Geology*, **35**(3), 253–278. doi:10.1023/A:1023866030544

Nash, D. R., & Boomsma, J. J. (2008). Communication between hosts and social parasites. In d'Ettorre P, & Hughes, D. P., (eds.), *Sociobiology of Communication: An Interdisciplinary Perspective*. Oxford University Press. doi:10.1093/acprof:oso/9780199216840.003.0004

Nehring, V., Dani, F. R., Turillazzi, S., Boomsma, J. J., & d'Ettorre, P. (2015). Integration strategies of a leaf-cutting ant social parasite. *Animal Behaviour*, **108**, 55–65. doi:10.1016/j.anbehav.2015.07.009

Neumann, P., & Moritz, R. (2002). The Cape honeybee phenomenon: The sympatric evolution of a social parasite in real time? *Behavioral Ecology and Sociobiology*, **52**(4), 271–281. doi:10.1007/s00265-002-0518-7

Neumann, P., & Pirk, C. W. W. (2019). Increased response to sequential infections of honeybee, Apis mellifera scutellata, colonies by socially parasitic Cape honeybee, *A. m. capensis*, workers. *Scientific Reports*, **9**(1), 7582. doi:10.1038/s41598-019-43920-1

Nishide, Y., Satoh, T., Hiraoka, T., Obara, Y., & Iwabuchi, K. (2007). Clonal structure affects the assembling behavior in the Japanese queenless ant *Pristomyrmex punctatus*. *Naturwissenschaften*, **94**(10), 865–869. doi:10.1007/s00114-007-0267-6

Ozaki, M., Wada-Katsumata, A., Fujikawa, K., Iwasaki, M., Yokohari, F., Satoji, Y., Nisimura, T., & Yamaoka, R. (2005). Ant nestmate and non-nestmate discrimination by a chemosensory sensillum. *Science*, **309**(5732), 311–314. doi:10.1126/science.1105244

Pull, C. D., & McMahon, D. P. (2020). Superorganism Immunity: A Major Transition in Immune System Evolution. *Frontiers in Ecology and Evolution*, **8**. doi:10.3389/fevo.2020.00186

R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. *http://www.r-project.org/*.

Rabeling, C. (2021). Social Parasitism. Pp. 836–858 in: Starr, C. K. (ed.), *Encyclopedia of Social Insects*. Springer International Publishing. doi:10.1007/978-3-030-28102-1_175

Richard, F.-J., & Hunt, J. H. (2013). Intracolony chemical communication in social insects. *Insectes Sociaux*, **60**(3), 275–291. doi:10.1007/s00040-013-0306-6

Roulston, T. H., Buczkowski, G., & Silverman, J. (2003). Nestmate discrimination in ants: Effect of bioassay on aggressive behavior. *Insectes Sociaux*, **50**(2), 151–159. doi:10.1007/s00040-003-0624-1

Sanada-Morimura, S., Minai, M., Yokoyama, M., Hirota, T., Satoh, T., & Obara, Y. (2003). Encounter-induced hostility to neighbors in the ant *Pristomyrmex pungens*. *Behavioral Ecology*, **14**(5), 713–718. doi:10.1093/beheco/arg057

Sasaki, T., & Tsuji, K. (2003). Behavioral property of unusual large workers in the ant, *Pristomyrmex pungens*. *Journal of Ethology*, **21**(2), 145–151. doi:10.1007/s10164-002-0085-4

Satow, S., Satoh, T., & Hirota, T. (2013). Colony fusion in a parthenogenetic ant, *Pristomyrmex punctatus*. *Journal of Insect Science*, **13**(1), 38. doi:10.1673/031.013.3801

Sturgis, S., & Gordon, D. (2012). Nestmate recognition in ants (Hymenoptera:

Formicidae): a review. *Myrmecological News*, **16**, 101–110.

Suarez, A. V., Tsutsui, N. D., Holway, D. A., & Case, T. J. (1999). Behavioral and genetic differentiation between native and introduced populations of the Argentine ant. *Biological Invasions*, **1**, 43–53. doi:10.1023/A:1010038413690

Tsuji, K. (1988a). Inter-colonial incompatibility and aggressive interactions in *Pristomyrmex pungens* (Hymenoptera: Formicidae). *Journal of Ethology*, **6**(2), 77–81. doi:10.1007/BF02350871

Tsuji, K. (1988b). Obligate parthenogenesis and reproductive division of labor in the Japanese queenless ant *Pristomyrmex pungens*: comparison of intranidal and extranidal workers. *Behavioral Ecology and Sociobiology*, **23**(4), 247–255. doi:10.1007/BF00302947

Tsuji, K. (1990a). Kin recognition in *Pristomyrmex pungens* (Hymenoptera: Formicidae): asymmetrical change in acceptance and rejection due to odour transfer. *Animal Behaviour*, **40**(2), 306–312. doi:10.1016/S0003-3472(05)80925-X

Tsuji, K. (1990b). Reproductive division of labour related to age in the Japanese queenless ant, *Pristomyrmex pungens*. *Animal Behaviour*, **39**(5), 843–849. doi:10.1016/s0003-3472(05)80948-0

Tsuji, K., & Dobata, S. (2011). Social cancer and the biology of the clonal ant *Pristomyrmex punctatus* (Hymenoptera: Formicidae). *Myrmecological News*, **15**, 91–99.

van Zweden, J. S., & d'Ettorre, P. (2010). Nestmate recognition in social insects and the role of hydrocarbons. Pp. 222–243 in: Bagnères, A.-G. & Blomquist, G. J. (eds.), *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology*. Cambridge University Press. doi:10.1017/CBO9780511711909.012

Wilson, E. O. (1971). *The Insect Societies*. Belknap Press of Harvard University Press.

Wilson, E. O. (1975). *Sociobiology: The New Synthesis*. Belknap Press of Harvard U Press.

Yagound, B., Dogantzis, K. A., Zayed, A., Lim, J., Broekhuyse, P., Remnant, E. J., Beekman, M., Allsopp, M. H., Aamidor, S. E., Dim, O., Buchmann, G., &

Oldroyd, B. P. (2020). A single gene causes thelytokous parthenogenesis, the defining feature of the Cape honeybee *Apis mellifera capensis*. *Current Biology*, **30**(12), 2248-2259. doi:10.1016/j.cub.2020.04.033

538 ## Tables and Figures

539 **Table 1**. Combination of source colonies in the colony introduction assay.

host-source intruder-source	A	B	C	D	F
H	✓			✓	✓
I	✓	✓			✓
J	✓	✓	✓		
K		✓	✓	✓	
M			✓	✓	✓

540

541 For each combination with a tick symbol, one cheater, one forager, and one nurse were
542 introduced.

543

Figure 1. An experimental host colony with an intruder (arrowhead) being introduced. Scale bar: 20 mm.

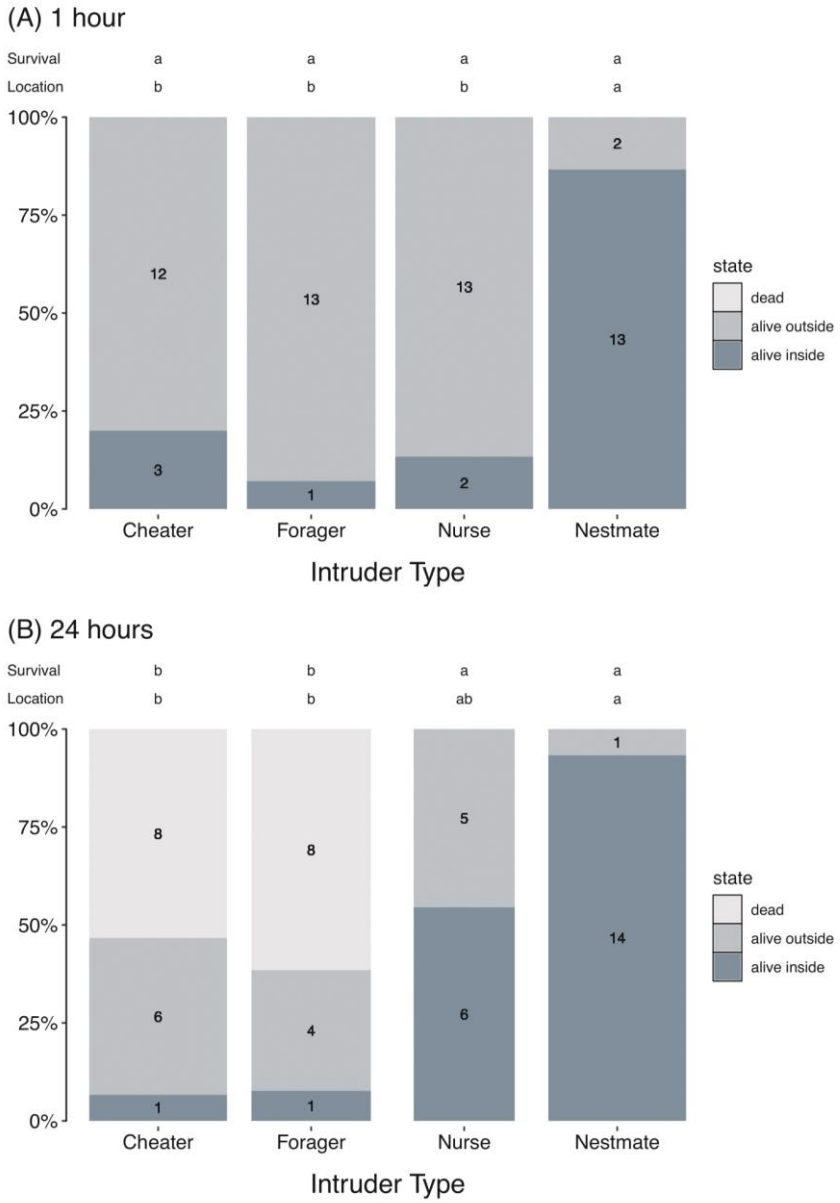

Figure 2. Results of colony introduction experiments. (A) after 1 hour and (B) after 24 hours of introduction. Bar width corresponds to the total number of trials. Different letters at the top indicate statistically significant differences among intruder types. Numbers in the bars indicate the number of individuals in each state.

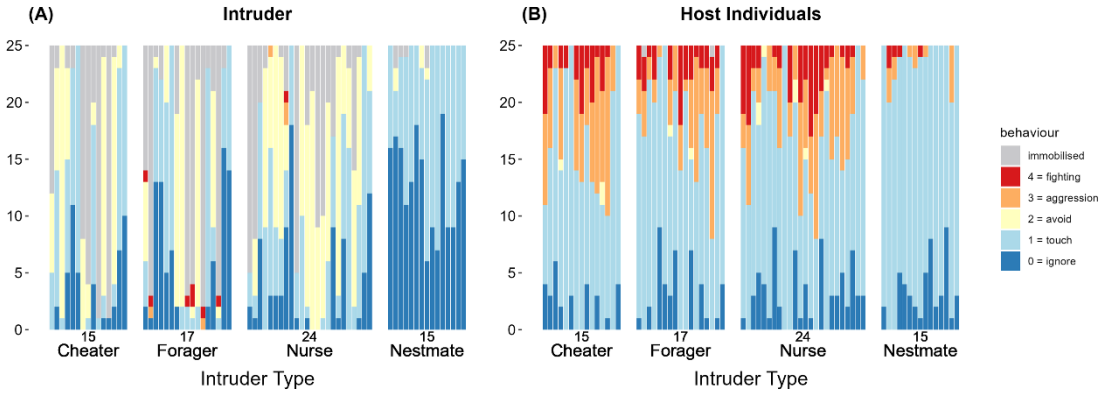

Figure 3. Frequencies of behavioural categories exhibited by (A) intruders and (B) host individuals in colony introduction experiments. Each vertical bar corresponds to an introduction trial, with their left-to-right arrangement indicating the same trial between (A) and (B).