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Social enforcement depending on the stage of colony growth in an ant

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Altruism is a paradox in Darwinian evolution. Policing is an important mechanism of the evolution and maintenance of altruism. A recently developed dynamic game model incorporating colony demography and inclusive fitness predicts that, in hymenopteran social insects, policing behaviour enforcing reproductive altruism in group members depends strongly on the colony growth stage, with strong policing as the colony develops and a relaxation of policing during the reproductive phase. Here, we report clear evidence supporting this prediction. In the ant *Diacamma* sp., reproduction by workers was suppressed by worker policing when the colony was small, whereas in large, mature colonies worker policing was relaxed and worker-produced males emerged. Conditional expression of traits can provide strong empirical evidence for natural selection theory if the expression pattern is precisely predicted by the theory, and our results illustrate the importance of intracolony population dynamics in the evolution of social systems.

1. Introduction

Organisms have evolved various life-history strategies to maximize their fitness in diverse environments [1,2]. Natural selection favours the optimal switching of resource allocation from growth to reproduction within an organism's lifetime [3–5]. This framework has been applied to social insect colonies, in which growth refers to an increase in colony size and reproduction the emergence of new reproductive offspring [6]. In many social Hymenoptera (ants, some bees and some wasps), workers are usually sterile [7]. This sterility is often partial, however, because workers cannot mate but can directly produce haploid males [7]. This partial sterility provides an opportunity to test a new theory that combines the frameworks of life-history strategy and kin selection [8].

Despite this potential for producing sons, direct reproduction by workers is rare except in orphan colonies [7]. Recent studies suggest that this reproductive altruism in workers is, to a large extent, a trait enforced by worker policing, defined as the inhibition of worker reproduction by other workers [9]. According to relatedness theory [10], when workers are more closely related to the queen's sons than to other workers' sons, they police other workers' reproduction. Comparative studies of many taxa have tested and partially supported this theory [11], but the bulk of interspecific variation remains unexplained [11,12]. In particular, the relatedness condition holds true only under polyandry (queen multiple mating) or polygyny (multiple related queens), whereas there are numerous examples of worker policing in monandrous (singly mated queen) and monogynous (single-queened) colonies.

Ohtsuki & Tsuji [8] provided a new hypothesis on worker policing; this hypothesis can explain its occurrence under monogyny and monandry. They incorporated intracolony population dynamics in an inclusive fitness model and predicted that worker policing will be conditional on the colony growth stage. Eusocial insect colonies usually produce only workers until reaching a certain colony size (the ergonomic stage) and then switch to producing sexual offspring (the reproductive stage), a strategy that is theoretically predicted to maximize the reproductive output of the entire colony [6]. Ohtsuki & Tsuji [8] predicted that reproduction by workers, which produces only males that do not contribute to colony maintenance and growth, should be strongly policed at the ergonomic stage, because producing only workers benefits all colony members in terms of lifetime inclusive fitness. In the reproductive stage, however, the model predicts that worker policing will be relaxed and worker-derived males can emerge when workers are not more closely related to the queen's sons than to other workers' sons (monogyny and monandry is the typical case for this). Note that this complex prediction results from the simultaneous operation of intracolonial conflicts over sex allocation, male parentage and reproductive allocation, which are usually considered separately by inclusive fitness models [7].

Importantly, what Ohtsuki & Tsuji [8] predicted was a facultative expression of social traits depending on the intra-colony population size. Conditional expression of traits can provide strong empirical evidence for natural selection theory, if the expression pattern is precisely predicted by the theory [13–15]. In testing such predictions, an approach focusing on a single species and the gathering of detailed biological information on the focal species might be more appropriate than cross-species comparisons. Thus far, empirical studies on worker policing that have taken this approach are still limited [16–20]. In this study, we empirically tested Ohtsuki and Tsuji's predictions, using the ant *Diacamma* sp.

2. Material and methods

(a) Ant species

Diacamma sp. from Japan is the only member of this genus distributed in Japan. Colonies have no morphological queen caste, and a mated worker, called a gamergate [21], reproduces as the functional queen. Each colony has, at most, a single gamergate (monogynous) that is usually singly mated (monandrous) ([22], but see also [23]). Reproductive specialization occurs at the adult stage by social manipulation [22]. All workers emerge with a pair of small thoracic appendages called gemmae [24], but only the gamergate will retain the gemmae throughout her life. The gamergate, and sometimes workers, bite and remove the gemmae of all callow workers soon after their emergence. Such mutilated workers lose their mating ability and become non-reproductive helpers. When the colony has lost the gamergate, a callow worker (usually the one that first emerges) is not mutilated, eventually mates, and becomes the new gamergate [22]. Once mutilated, a worker can never be the gamergate, but she retains the ability to lay male-destined haploid eggs [25]. Such worker reproduction is rare when the gamergate is present in the colony but is frequent in orphan colonies [26]. *Diacamma* sp. colonies contain 30 to 300 workers in the field [27], and males usually emerge from colonies containing 100 workers or more ([28], see also the electronic supplementary material, figure S3). Relatedness among workers can be affected (lowered) by recent gamergate turnover, but it is not actually correlated with colony size (see Results). We use the term 'queen' instead of 'gamergate' in this paper.

All colonies were collected between 1997 and 2008 in Nakijin on Okinawa Island, Japan. Each colony was housed in a standard artificial nest [29] and kept in the laboratory (26 ± 1°C, 14 L : 10 D cycle). They were fed ad libitum on mealworms and water.

(b) Split-fusion experiment

To evaluate the efficiency of worker policing via cannibalism of worker-produced eggs, we applied a colony splitting and fusion procedure described previously [30]. We split a colony into two types of subcolonies with an equal number of workers: one subcolony contained a queen and the other only workers, without any brood. This latter manipulation can induce worker reproduction in queen-absent subcolonies. We continuously observed each colony for 12 h after colony re-unification and recorded all incidences of worker reproduction and egg destruction by other adults (workers and the queen) in eight queen-right colonies (i.e. colonies with a surviving queen) of various sizes (92 to 218 workers). We scored a worker-produced egg as having 'survived' if it was transported to an egg pile, as egg destruction after this stage is seemingly rare [31]. We used a generalized linear model (GLM) with a binomial error structure to examine the effect of colony size on the fate of worker-produced eggs.

(c) Egg-introduction experiment

In the split-fusion experiment, it was possible that the number of reproductive workers would differ among experimental colonies. The proportion of reproductive workers to policing workers can affect the efficiency of worker policing [32]. To circumvent this difficulty, we performed an egg-introduction experiment. We applied the method of egg introduction described previously [31], using seven colonies (approx. 100–130 workers) as the sources of queen-produced and worker-produced eggs (donor colonies). We also prepared another set of 10 colonies of various sizes (41–190 workers) to which eggs were introduced (recipient colonies). Each donor colony was split into half-sized subcolonies, namely the queen-right colony and the orphan one. We collected eggs from the orphan subcolony for a week after worker reproduction had begun in the colony. To obtain the queen-laid eggs, we temporarily isolated the gamergate with a forager worker in a glass tube (foragers do not have functional ovaries [25]). All eggs were collected within 12 h after being laid, and each egg was introduced to a randomly chosen recipient colony. We used forceps to place the egg on a glass slide, which was then placed near (less than 2 cm) the nest entrance of a recipient colony. We introduced 24, 14, 20, 19, 24, 21, 21, 21, 30 and 22 worker-produced eggs into experimental colonies containing 41, 45, 65, 91, 110, 137, 154, 172, 179 and 190 workers, respectively. We also introduced 5, 9, 9, 9, 12, 8, 7, 8, 6 and 3 queen-produced eggs, respectively, into the above experimental colonies. All introduced eggs were soon found by a worker and transferred into the nest. We observed the fate of each egg for 30 min after it was transferred. If the focal egg was not destroyed during this observation period, we regarded the egg as accepted. No additional egg was introduced into the nest before we had completed each observation period. We used a GLM with a binomial error distribution to test the effect of size of the recipient colony on the eggs' fates.

(d) Colony size and relatedness in the field

Although *Diacamma* sp. is reported to be monogynous and monandrous [22], frequent queen turnover can decrease genetic relatedness, as in polygynous colonies. Furthermore, if kin structure differs depending on the colony size, the strength of policing can also depend on colony size, thus violating the assumption of the model of Ohtsuki & Tsuji [8]. To investigate the relationship between genetic structure and colony size, we estimated the mean relatedness among workers in 17 colonies of various sizes (24–213 workers) collected in October 2005. Immediately after the field collection, we sampled 15 workers from each colony and determined their genotypes at four polymorphic microsatellite loci (see 'Genetic analyses' section for details). The average relatedness among nest-mate workers was estimated for

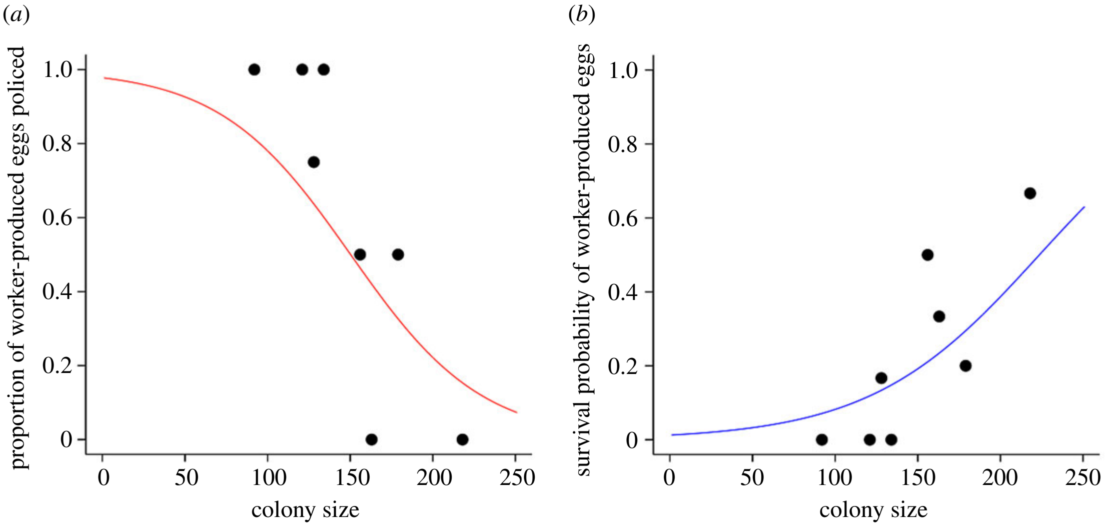

Figure 1. Colony size versus the fate of worker-produced eggs in the split-fusion experiment. (*a*) Proportions of eggs destroyed by workers among worker-produced eggs not destroyed by the queen. (*b*) Survival probabilities of worker-produced eggs. Each point corresponds to an experimental colony, and regression curves were obtained from generalized linear models.

each colony by using the software RELATEDNESS v. 4.2 [33]. The correlation between colony size and mean relatedness among workers was tested by the Kendall rank correlation test and the bootstrap test using Kendall's τ (10 000 permutations).

(e) Male parentage

We estimated the proportion of worker-produced adult males by using five laboratory colonies. Because queen turnover can occur in this ant [22], field colonies may contain non-sister workers that are daughters of the previous queen(s). We manipulated the colonies to contain only sister workers in the following way. Shortly after the field collection of colonies, we pseudo-randomly chose 50 workers and queens and removed all brood (non-adults). All those adults were individually marked with enamel paint. After laboratory rearing for eight to 12 weeks, we removed all marked workers and then reared those colonies, now consisting of only sister workers and the queen, for up to 12 months. All males produced were collected and genotyped by using the four microsatellite marker loci, and at the end the queen and 10–15 workers per colony were also genotyped. Few males emerged (5–12 individuals per colony), possibly because of the captive conditions. Furthermore, the low variation in marker loci hindered the determination of maternity of all males. Therefore, we used maximum-likelihood estimation to extrapolate the origin of males [34].

(f) Genetic analyses

The head of each individual was separately crushed in digestion solution (10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 25 mM NaCl, 200 μg ml^{-1} proteinase K) and incubated for 2 h at 50°C and then at 90°C for 15 min. DNA solution was used as template for polymerase chain reaction (PCR) after being diluted with 10 volumes of Tris-EDTA buffer. PCR amplifications were carried out in a total volume of 13 μl, which contained 1.3 μl of template DNA, 0.5 μM of each microsatellite primer, 200 mM of deoxynucleotide, 1 × reaction buffer with $MgCl_2$ and 0.06 U of AmpliTaq Gold (Applied Biosystems). After an initial denaturing step of 10 min at 95°C, the PCR consisted of 40 cycles of 30 s at 96°C, 30 s at 58°C, and 60 s at 74°C, followed by a final extension step of 10 min at 74°C (MJ Research PTC-200 or ASTEC PC-818 thermocycler). The genotypes were determined using a CEQ 8800 sequencer (Beckman). We determined the genotypes at four polymorphic microsatellite loci that were informative in the colony: DC-8 [35], Plu-24 [36], DI33 [37] and DspS554 [24].

3. Results

(a) Split-fusion experiment

To examine the relationship between the intensity of worker policing and colony size, in the colony split-fusion experiment we first experimentally induced worker reproduction by isolating a portion of the workers for a while from the effects of pheromonal inhibition by the queen. Workers laid 16, 16, 6, 4, 2, 6, 20 and 3 eggs in experimental colonies containing 92, 121, 128, 134, 156, 163, 179 and 218 workers, respectively. The proportion of worker-produced eggs policed by workers (among those not destroyed by the queen) was negatively correlated with the colony size (GLM, $z = -2.569$, $p = 0.0102$; figure 1*a*). The resultant survival probability of worker-produced eggs was positively correlated with colony size (GLM, $z = 2.862$, $p = 0.0042$). No worker-produced egg survived in the smallest two colonies (figure 1*b*). We also conducted GLM analyses using a dataset excluding the largest colony of 218 workers, considering it as a possible outlier. This analysis indicated a similar trend as that of the results using the original data (proportion of worker-produced eggs policed by workers: GLM, $z = -2.410$, $p = 0.0160$; survival rate of worker-produced eggs: GLM, $z = 2.283$, $p = 0.02243$). Note that queen-produced eggs were never destroyed over the course of this experiment.

(b) Egg-introduction experiment

A similar pattern was observed in the egg-introduction experiment used to test the relationship between the intensity of worker policing and colony size. Worker-produced eggs were more likely to be policed by workers when they were introduced into small colonies than into large colonies (GLM, $z = -4.311$, $p < 0.0001$; figure 2*a*). The survival probability of worker-produced eggs was positively correlated with colony size (GLM, $z = 3.805$, $p < 0.0001$; figure 2*b*). Together, these results strongly support the prediction of Ohtsuki and Tsuji's model [8].

Worker policing was directed more strongly towards worker-produced eggs (139 out of 185, 75%) than to queen-produced eggs (23 out of 61, 38%; generalized linear mixed model with a binomial error structure, $\chi^2 = 7.385$, $p = 0.007$). This

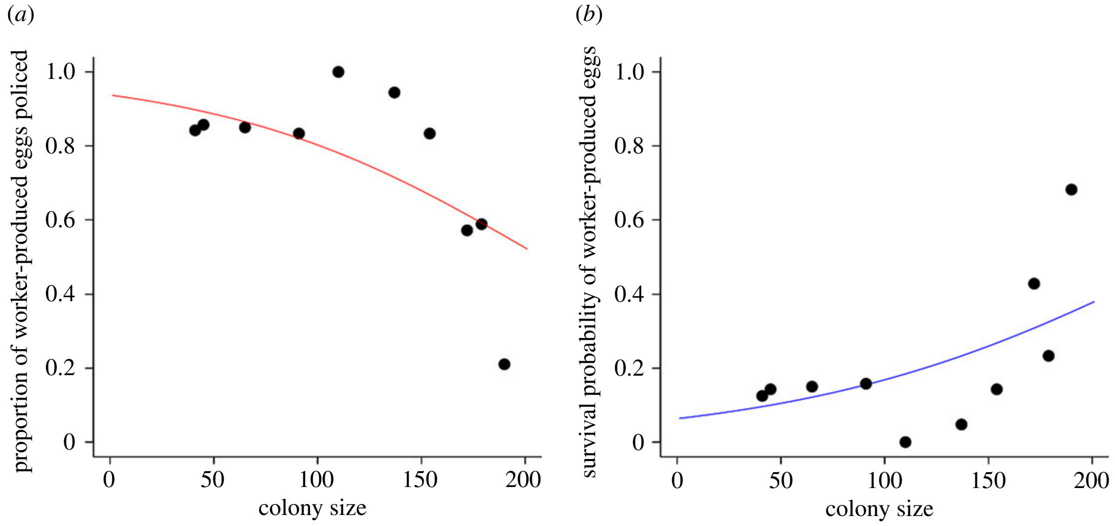

Figure 2. Colony size versus the fate of introduced worker-derived eggs in the egg-introduction experiment. (*a*) Proportions of eggs destroyed by workers among worker-produced eggs not destroyed by the queen. (*b*) Survival probabilities of worker-produced eggs over a 30 min period. Each point corresponds to an experimental colony, and regression curves were obtained from generalized linear models.

suggests that *Diacamma* sp. workers can distinguish the caste (queen or worker) of the egg's mother and express policing behaviour even if the eggs encountered are foreign; this supports the findings of Shimoji *et al*. [31]. No correlation of colony size with the proportion of queen-produced eggs policed by workers (GLM, $z = -0.565$, $p = 0.572$; electronic supplementary material, figure S1*a*) or with survival of queen-produced eggs (GLM, $z = -0.992$, $p = 0.321$, electronic supplementary material, figure S1*b*) was detected. Here, also, we conducted GLM analyses using a dataset without the largest colony (190 workers), considering it as a potential outlier. These results were similar to those using the original data (proportion of worker-produced eggs policed by workers: GLM, $z = -2.427$, $p = 0.0152$; survival rate of worker-produced eggs: GLM, $z = 1.932$, $p = 0.053$).

(c) Colony size and relatedness in the field

To test the relationship between genetic structure and colony size, we estimated relatedness within colony by each colony using polymorphic nuclear microsatellite markers. Our genetic analysis revealed that the average among-worker relatedness ($r = 0.55 \pm 0.5$, mean ± s.e.) was not significantly different from 0.5 (95% confidence interval: 0.45–0.66). Furthermore, if kin structure differs depending on colony size, then the strength of policing can also be colony-size dependent. Moreover, an association between relatedness and colony size would violate an assumption of Ohtsuki and Tsuji's model [8]. To clarify whether this is the case, we examined the relationship between relatedness among workers and colony size. Genetic analysis of field colonies revealed that colony size was not significantly associated with intracolonial relatedness among workers (Kendall rank correlation test, $\tau = -0.0735$, $p = 0.715$; estimation after bootstrap using τ (10 000 permutations), $p = 0.358$; electronic supplementary material, figure S2). Therefore, the genetic structure of colonies in *Diacamma* sp. satisfies the model's assumptions.

(d) Male parentage

Finally, we tested Ohtsuki & Tsuji's [8] second prediction, namely that worker-produced males would emerge. The genetic analysis and maximum-likelihood estimation revealed that, on average, 58.4% ± 12.7% (mean ± s.d.) of males were derived from workers (table 1). Because colonies used for this analysis consisted exclusively of the queen and her daughter workers, we can exclude the influence of previous queen turnover on the genetic composition of the colonies. Contrary to what was believed previously, double mating by a queen was detected in a colony. Worker-produced males ($n = 2$) also emerged in this colony.

Table 1. Proportions of worker-produced males, as estimated by using the maximum-likelihood method.

colony ID	no. males produced	estimated proportion
17	9	1.00
18	12	0.66
19	9	0.26
25	8	0.27
26	5	0.72

4. Discussion

Our data strongly supported two major predictions of Ohtsuki and Tsuji's model [8]. First, when workers were not more closely related to the queen's sons than to other workers' sons, as predicted, workers policed worker reproduction strongly at the ergonomic stage but policed it weakly at the reproductive stage. *Diacamma* sp. colonies enter the reproductive stage (i.e. when males are produced) when colony size exceeds about 100 workers, as discussed below. Indeed, the shift in policing strength occurred around this threshold colony size (figures 1 and 2). The estimated relatedness among workers (0.55 ± 0.5), which was not significantly different from 0.5, satisfied the model's assumption, because for a given worker the expected relatedness of other workers' sons (0.275) was not lower than the relatedness of the queen's sons (0.25 when the queen was the mother of the focal worker, and 0.275 when the queen was a sister of the focal worker owing to a recent queen turnover). Furthermore, because relatedness was not significantly correlated with colony size (electronic supplementary material, figure S2), the observed variation in policing strength was unlikely to reflect a difference in relatedness. Second, worker-produced males were predicted to emerge. Indeed, we found that more than half the males emerging (58%) were likely to be worker-produced.

Nakata & Tsuji [28] reported that *Diacamma* sp. colonies entered the reproductive stage when their colony size exceeded 150 workers. Because this is a critical assumption of the current study, we also tested it, using our male parentage data. We obtained 56 males from five colonies, including those excluded from the genetic analysis (see Material and methods). The majority of males (47 out of 56) emerged in colonies containing more than 100 workers, whereas no male (i.e. 'early male' [38]) emerged in small colonies with fewer than 50 workers (electronic supplementary material, figure S3). Thus, our observations also suggest that the switch from ergonomic to reproductive stage occurs in *Diacamma* sp. colonies. But unlike in the study by Nakata & Tsuji [28], under our experimental conditions colonies appeared to gradually switch resource allocation from colony maintenance (worker production) to reproduction (male production) when the colony size exceeded about 100 workers. Note, however, that this observation seemed to be consistent with Ohtsuki and Tsuji's hypothesis, because policing strength also gradually declined as colony size increased (figures 1 and 2).

Empirical evidence shows that policing is widespread in social insects [9,11,20,39]. Two hypotheses have been proposed for the evolutionary driving force underlying worker policing. The relatedness hypothesis [10] predicts that workers should prevent other workers from laying male-destined eggs when workers are more closely related to the queen's sons than to other workers' sons. Evidence supporting this hypothesis has been reported for many species [11]. Alternatively, the cost hypothesis predicts that, if worker reproduction harms colony efficiency [40,41], worker policing and self-restraint of worker reproduction can evolve [11]. Empirical data supporting this hypothesis are also accumulating [30,42,43]. Ohtsuki and Tsuji's insight was that both relatedness and costs can work as the evolutionary driving forces, but their relative importance changes depending on the colony growth stage: that is, cost is more important at the ergonomic stage, whereas relatedness becomes more important at the reproductive stage. However, thus far, few empirical studies have examined the relationship between policing and a colony's demographic state. In the parthenogenetic ant *Ooceraea biroi*, worker policing occurs to synchronize the brood development that determines the colony state (migratory or stationary) [20]. In the Saxon wasp, *Dolichovespula saxonica*, the proportion of worker-produced males is positively correlated with colony size [17], and in the ant *Temnothorax unifasciatus* the strength of worker policing changes with the colony size [19]. These results partially support the prediction of Ohtsuki & Tsuji [8], but simultaneous examination of male parentage and policing behaviour is still to be done. Although the effect of colony growth stage on policing has been studied in *Camponotus floridanus* [18], that study did not directly test Ohtsuki and Tsuji's model, because it compared colonies at the incipient (very small) stage and those at the ergonomic stage. Thus, the current study provides the strongest empirical evidence supporting the model.

Our current results indicate that, despite recent discussions to the contrary [44], the inclusive fitness model is a powerful tool for predicting the facultative expression of social behaviours such as worker policing, provided that the background population dynamics are appropriately taken into account. In organisms other than social insects, population dynamics have long been discussed as a factor affecting the expression of animal behaviours such as aggression, migration and even altruism [45,46]. Future studies should be more aware of the potential importance of demography in social evolution.

Many questions remain with regard to the proximate mechanisms underlying worker policing in *Diacamma* sp. For example, how do ants sense the growth stage of the colony, and what cues trigger behavioural and physiological changes in individual workers? *Diacamma* sp. queens also respond behaviourally to changing colony size [27]. In the reproductive division of labour in *Diacamma* sp., there is evidence of the involvement of biogenic amines [47,48] and gene expression [49]. Future research should endeavour to combine these pieces of knowledge for a more complete understanding, as joining intracolony population dynamics and inclusive fitness provides new insights into worker policing.

Data accessibility. This article has no additional data.

Authors' contributions. H.S., T.K., N.K. and K.T. designed the study and performed the behavioural experiments. H.O. carried out genetic analyses. H.S. and K.T. wrote the manuscript. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

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