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Paint marking using CO_2 anaesthetization does not affect exploratory and recruitment behaviours in the rock ant, *Temnothorax rugatulus*

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A R T I C L E I N F O

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A B S T R A C T

The study of animal behaviour sometimes requires unique identification of individuals, especially in the study of social behaviours involving the interactions of multiple individuals. To this end, researchers have developed many different methods of marking individuals. For small animals like insects, paint marks are often applied to their bodies by anaesthetizing them using low temperature or carbon dioxide. Despite this procedure being ubiquitous when studying social insects, the effect of paint and anaesthetics on their behaviour has not been well investigated, especially their effect on performance during a collective task. In our study, we investigate how paint marks and anaesthetics affect the movement and recruitment behaviours of the ant *Temnothorax rugatulus* in a house hunting context. We painted two thirds of colony members, half of them using CO_2 and the other half using low temperature as methods of anaesthetization, and left the one third unpainted as a control group. We then measured their exploratory behaviour prior to house hunting and their recruitment behaviours during house hunting. We found that neither paint marks nor anaesthetics reduce activity levels of these behaviours. However, low-temperature anaesthetized ants performed a *higher* number of recruitment behaviours than control ants. Because CO_2 anaesthetized ants performed all tasks at the same level as control ants, our data suggest that this is a good technique for paint marking ants, especially *T. rugatulus*. This is the first study empirically testing negative effects of paint marking on individual and collective outcomes in social insects. Our study represents an important step towards routine validation of individual identification methods used in the study of animal behaviour.

Animal identification is essential for the study of animal behaviour. By identifying and tracking individuals, researchers are able to obtain valuable behavioural information, including home ranges (Kalan et al., 2016; Rimmer et al., 2017), resource choice (Bartelt et al., 2004; Garrison et al., 2016; Weber and Lundgren, 2009), social behaviours (Blondel, 2006; Godfrey et al., 2010), and movement behaviours (Boina et al., 2009; Gillies, 1961), of various animal species. This detailed information about individual actions also allows researchers to investigate mechanisms of collective patterns observed in animal groups. For example, researchers were able to create social networks of the territorial reptile tuatara by marking individuals and investigating if and how the network structure is related to parasite transmission (Godfrey et al., 2010). Thus, individual identification helps advance our understanding of individual and collective behaviour of animal groups (Charbonneau et al., 2017; Wilson-Rankin, 2015; Yang et al., 2009).

The ability to track individuals is especially important to investigate collective behaviour of social insects, which exhibit highly organized societies (Hölldobler and Wilson, 2009). Individual identification allows researchers to decipher complex phenomena including self-organization, division of labour (Huang and Robinson, 1992; Ingram et al., 2005) and collective decision making (Sasaki and Pratt, 2018). For example, by tracking all the individual ants in a colony, researchers are able to look at how tasks are divided among workers (Charbonneau and Dornhaus, 2015; Pinter-Wollman et al., 2012). Furthermore, when specific (e.g. highly active) workers are removed from their colonies, other (e.g. inactive) workers may replace them and maintain collective performance (Charbonneau et al., 2017). These individual-tracking studies have revealed that, although colony members appear similar, they are in fact heterogeneous, which is an important concept for probing various complex phenomena observed in social insects (Charbonneau et al., 2017; Jandt et al., 2014).

Researchers have developed various techniques, such as mutilation,

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tags or bands, polymers and pigments, and radio transmitters (Murray and Fuller, 2000; Southwood, 1978a) for tracking individual animals. These techniques are typically used to mark vertebrates, and studies have examined how these marking methods affect the behaviour and welfare of the marked animals (Bodey et al., 2018; Langkilde and Shine, 2006; Petit et al., 2012; Soulsbury et al., 2020). To track small animals, such as insects, the most common technique is the use of paint or inks (Southwood, 1978b; Walker and Wineriter, 1981), where insect bodies are marked with unique combinations of colours (Fig. 1). While this paint marking is an essential process for identifying individuals, the paint itself may affect an individual's behaviour. For example, a study on termites has shown that paint marking can change their foraging movement. Individuals covered with more dorsal paint travel farther when foraging (Nagendra et al., 2010). Furthermore, painted social wasps groom each other more often than do unpainted ones (De Souza et al., 2012). While this paint marking technique has been widely used for individual tracking in social insects, studies investigating the effect of paint marks on their behaviour are limited to only a few species. Also, researchers have primarily focused on the effect on individual behaviours and dyadic interactions and its effect on collective outcomes has been largely overlooked.

The effect of the process to prepare for painting also has been under-investigated. To apply the paint, researchers commonly anaesthetize the insect using carbon dioxide or low temperature (Holbrook, 2009), which can affect their behaviour. For example, exposure to carbon dioxide in bumblebees caused individuals to spend less time caring for brood and increase their activity levels (Poissonnier et al., 2015). In *Apis mellifera*, individuals exposed to carbon dioxide modify their hoarding behaviour and reduce pollen gathering (Nicolas and Sillans, 1989). Cold anaesthesia also affects the behaviour of different species; for example, studies show that *Bombus occidentalis* have a lower foraging recruitment rate (Wilson et al., 2006), *Bombus terrestris* spend less time foraging and higher nestmate aggression (Poissonnier et al., 2015) and *Apis mellifera* show impaired memory formation (Erber et al., 1980). Most studies on effects of anaesthesia in social insects have focused on bees. To our knowledge, there is only one study looking at the effect of low temperature in ants. *Formica xerophila* displays higher interspecific and nestmate aggression when exposed to low temperatures compared to unexposed ants (Tanner, 2009). It is thus important to understand the effects of different methods of anaesthetization on ants, as these processes can affect their individual behaviour as well as collective behaviour.

This study aims to investigate how paint marks and different methods of anaesthetization affect individual movement patterns and participation in a collective task. To do so, we used the rock ant, *Temnothorax rugatulus,* which is a common model organism for the study of collective behaviour, especially their emigration behaviour (Dornhaus et al., 2008; Pratt et al., 2002; Sasaki and Pratt, 2012, 2018). These ants live in ephemeral nests, such as rock crevices, and frequently emigrate to new homes (Möglich, 1978). When the home nest is damaged, roughly one third of colony members leave the home and look for a new site (Pratt et al., 2002). Once these scouts find a desirable nest site, they go back home and recruit their nestmates one by one to the new site using tandem running or social transport (Möglich, 1978). These are direct recruitment mechanisms where a knowledgeable individual recruits a single naïve individual to the target one by one (Pratt, 2005). Through these recruitments, the whole colony eventually moves to the new site. In our experiment, we first measured exploratory behaviour of individual workers when the home nest was intact. We then induced an emigration by removing the roof of the home nest and observed their recruitment behaviours. We tested the hypotheses that painting and anaesthetization would have no effect on exploration and recruitment behaviour in *Temnothorax* colonies.

1. Methods

1.1. Colony collection and housing

We collected six queenright colonies of *T. rugatulus* ants from the Pinal Mountains near Globe, Arizona (N 33º19.00′N 110º52.56′W) on 24 May 2019. The average colony size was 119 ± 31 ants. The colonies were housed in artificial nests consisting of two glass microscope slides (7.5 cm × 5 cm) above and below a piece of cardboard (2 mm thick) with a nest cavity (5 cm × 2 cm) and entrance (1.5 cm × 0.4 cm) cut out (Sasaki and Pratt, 2012, p. 201). Artificial nests were placed inside 11 × 11 cm plastic boxes with walls coated with fluon anti-traction compound to prevent the ants from escaping. Colonies were kept in the lab inside an incubator maintained at 13 ℃ at 12:12 light:dark cycle and provided with water, canned pork product and Bhatkar's solution (Bhatkar and Whitcomb, 1970) ad libitum. All colonies were used only once.

Fig. 1. Painted *Temnothorax rugatulus* ants within a nest. The ants used in our experiment were similarly painted, but members in each treatment group shared the same colour mark. The unique paint marks shown in this picture are a common method for individual tracking in ants.

1.2. Marking method

To test the effects of the paint and anaesthesia on behaviours, we randomly chose two-thirds of ants in each colony as a ‘paint’ group and the rest as a ‘control’ group, which was left unpainted. Half of the ants in the paint group (i.e., one-third of the whole colony) were anaesthetized with CO_2 gas, and the other half were anaesthetized with low temperature. For the CO_2 anesthetization, we picked each ant up with soft forceps from the nest box and placed her in a small open cylinder (9 cm diameter; 4 cm height). A small tube continuously emitted CO_2 gas into this cylinder. The ant was exposed to the gas for the entire duration of painting to ensure she stayed immobile, which lasted around 3–4 min. For the low-temperature anesthetization, we placed each individual in a separate vial (1.5 cm diameter, 4 cm height) using soft forceps and transferred the vial to a freezer for 4–5 min at − 10 ℃ or 1–2 min at − 18 ℃. Once we confirmed that the ant was not moving, we placed her on a chilled sponge on a chill table (BioQuip Laboratory Chill Table) at -5 ℃ to ensure she stayed immobile when painting.

Once the ant was anaesthetized using either the CO_2 or low-temperature method, we harnessed her using a fine thread wrapped on a sponge made from hydrophilic foam. The immobilized ants were then painted by dipping a pinhead into paint (obtained from Sharpie® Oil-Based Paint Markers) and marking a dot on the head, thorax and abdomen of each individual. We chose oil based paints in our experiment because these paints are often used for marking ants due to their high durability (Walker and Wineriter, 1981). It typically took 3–4 min to complete painting each ant. After the painting, the secured ants were placed in front of a small fan for 5 min to facilitate paint drying, after which they were returned to their nest box. All individuals in each treatment group (i.e., the CO_2 group or the low-temperature group) were painted with the same colour (pink or white) and thus could not be visually distinguished from each other. The colour used for each treatment group was randomly assigned in each colony. The queens, the brood, and the ants in the control group were not painted. The mortality rate was very low (less than 5%) for both the treatment groups.

1.3. Experimental procedure

The experimental procedure took place 24 – 48 h after the painting was completed. There were two behavioural phases that we monitored in our experiment –exploration and emigration. For the exploration phase, the nest containing the colony was first removed from the nest box and gently placed on one side of an experimental arena (28 cm × 17 cm) with fluon coated walls (Fig. 2a). We then video-recorded the whole arena for one hour using a 4k camera (Sony FDR AX 100 Handycam). After this exploration phase, we started the emigration phase by placing a new nest as a target nest on the other side on the arena and removing the roof of the old nest (Fig. 2b). During this phase, we filmed the two nests closely using two additional cameras, one above each nest. The recording was stopped 30 min after no ants or brood items remained

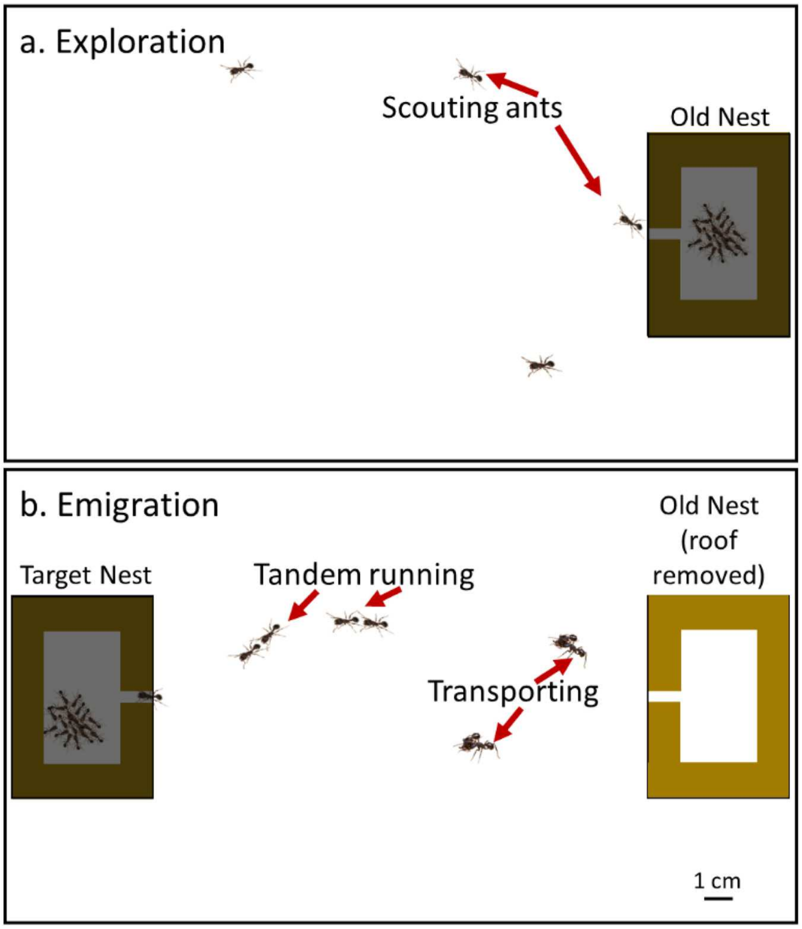

Fig. 2. Experimental arena during the (a) exploration phase and (b) emigration phase. In the exploration phase, a colony with a home nest was placed in the arena and the entire arena was recorded. In the emigration phase, the target nest was introduced to the arena, and the roof of home nest was removed to induce an emigration. The entire arena was filmed until all the ants and brood items were transported into the target nest.

at the old nest (4.43 hrs ± 2.633 hrs).

1.4. Video analysis

Thanks to the high-resolution 4 K cameras, we were able to identify the number of unique scouts (ants that were outside the nest) by manually following each ant in the video during the exploration phase. However, we were able to accurately follow scouts for only the first ten minutes due to difficulty in following multiple individuals, especially near nest entrance, for a long period of time. In the emigration phase, we were also able to identify the number of unique tandem-running and transport recruiters using the same method. As for the transport, however, we used only the first 30% of the total number of events for the analysis. This is because, when the number of ants in the target nest became high, they overlapped with each other near the entrance of the target nest so we were no longer able to accurately follow transport recruiters.

We also obtained the travel path of the recruiters by manually clicking the recruiter frame by frame (at one frame per second) using Fiji (Schindelin et al., 2012) with the MtrackJ plugin (Meijering et al., 2012). Because there were many transport events (mean = 118.83 ± 30.53), we were able to trace the first 10% of all transport recruiters due to the time constraint.

1.5. Statistical analyses

All statistical analyses were performed in R (Version 3.0.3).

1.6. Effect of painting and anaesthetization on exploration phase

We first looked at the effect of the treatment (painting and the method of anaesthetization) on whether an ant left the nest and explored the arena. We built generalized linear mixed-effect models (GLMM) using the lme4 package to test if the treatment affected the likelihood of ants leaving the nest and exploring the arena. Each GLMM was built with a binomial response (whether the ant left the nest or not). The fixed effect was the treatment (control, painted on anaesthetizing with CO2, and painted on anaesthetizing with low temperature) and the random effect was colony ID.

1.7. Effect of painting and anaesthetization on emigration phase

We first looked at the effect of the treatment on recruitment events conducted by each ant. Since only around 15–20% of a colony performed any recruitment event (tandem runs or transports) (Pratt, 2005), our data were zero inflated (i.e. most ants had zero events). In order to deal with this zero inflation, we built hurdle models using the glmmTMB package, which runs two separate regressions (Zuur et al., 2009). In the first regression, the response variable was binary (i.e., whether each ant performed at least one tandem run or transport), and we tested if the treatment affected whether ants performed tandem runs or transports. In the second regression, the response variable included only the ants that performed at least one tandem run or transport, and we tested if the treatment had the effect on the number of tandem run or transport events these recruiters performed. In both regressions, we used the treatment (unpainted, painted on anaesthetizing with CO_2 and painted on anaesthetizing with low temperature) as a fixed effect and colony ID as a random effect.

To analyse the effect of the treatment on the route efficiency of the tandem-run or transport path, we built a linear mixed-effects model (LMM) with nested random effects using the lme4 package. Our response variable was the efficiency (the distance of the straight line connecting the start and end points divided by that of the path walked by the ant) of the tandem run or transport route. Since each ant had a slightly different start and end point, using efficiency instead of walking distance as our response variable allowed us to standardize across these variations in the route. The fixed effect was the treatment group, and the random effects were the identity of the ant performing the recruitment event and the identity of the colony that ant belonged to. Ant identity was used as a random effect as there were situations where a single ant performed multiple tandem runs/transports. These ants were grouped by colony identity. We compared this model to a null model with no predictors to test if treatment affected the route efficiency using a likelihood ratio test.

1.8. Ethical note

The data were collected under the University of Georgia's ethics guideline on animal handling and welfare. Ants are not covered by the Institutional Animal Care and Use Committee (IACUC) guidelines.

2. Results

2.1. Effect of painting and anaesthetization on number of ants exploring the arena

GLMMs showed that there was no significant difference in the number of ants that performed at least one trip outside the nest, when comparing control ants with CO_2 anaesthetized ants ($Z = -0.611$, $p = 0.54$) and with low temperature anaesthetized ants ($Z = -0.358$, $p = 0.72$). These results indicate that painting and its preparatory procedures do not affect the probability that an ant leaves the nest and explores the areas surrounding it (Fig. 3).

2.2. Effect of painting and anaesthetization on number of recruiters during emigration

The hurdle models showed that there was no significant difference in the number of ants that performed at least one tandem run, when

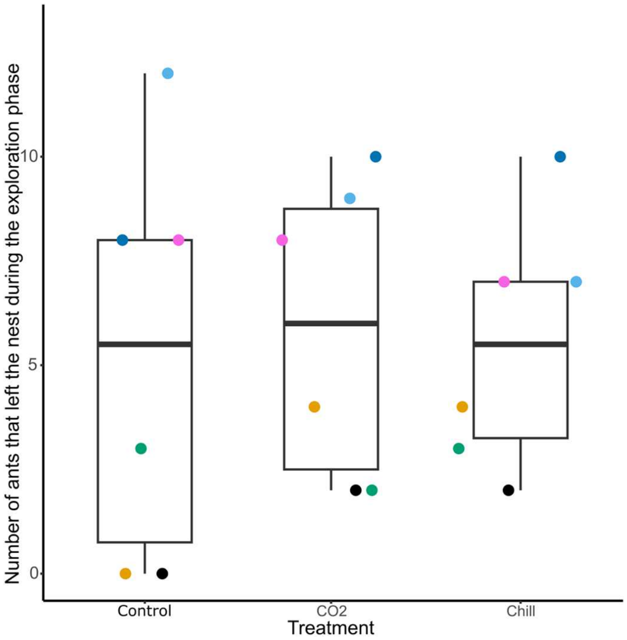

Fig. 3. Box plots showing the number of ants in each treatment group that left the nest during the exploration phase. Each point represents the number of "exploratory" ants in each treatment group for a colony, and the colour represents the colony identity. Each box extends between the lower and upper quartiles; the horizontal line within the box indicates the median, and whiskers show the range of the data (n = 6 colonies).

comparing the control group with the CO_2 anaesthetized group ($Z = 0.428$, $p = 0.67$), and with the low temperature anaesthetized group ($Z = 1.184$, $p = 0.24$). A similar pattern was observed for the transport recruitment acts (control vs. CO_2: $Z = 0.7$, $p = 0.48$, control vs. low temperature: $Z = -1.7$, $p = 0.09$). These results indicate that painting and its associated procedures do not affect the probability that the ant performs a tandem run or transport (Fig. 4).

2.3. Effect of anaesthetization on number of tandem-run events during emigration

Treatment affected the number of recruitment events that recruiter ants performed. Interestingly, recruiters that were marked by anaesthetizing with low temperatures performed more tandem runs than unpainted recruiters (truncated Poisson GLM, $Z = 2.5$. $p = 0.01$). This pattern was also observed in the case of the transport behaviour ($Z = 2$, $p = 0.04$). CO_2 did not have an effect on the number of tandem running ($Z = 1.24$, $p = 0.22$) and transport ($Z = 1.25$, $p = 0.21$) events (Fig. 5).

2.4. Effect of painting and anaesthetization on route efficiency of recruitment events during emigration

Linear-mixed effect models showed that there was no significant difference among treatments in the route efficiency (the distance of the straight line connecting the start and end points divided by that of the path walked by the ant) of tandem runs ($\chi^2(2) = 1.066$, $p = 0.59$) or transports ($\chi^2(2) = 0.037$, $p = 0.98$). These results indicate that painting and the different anaesthetization methods did not affect task performance for the ants that engage in tandem running or transport behaviours (Fig. 6).

3. Discussion

Paint marking is a common technique used by researchers to study the behaviour of insects, especially group living insects where it is necessary to distinguish among multiple individuals. Despite the ubiquity of this technique, the effects of painting have rarely been studied. In this paper, we investigated if a common method of marking individual ants – anaesthetizing and then placing paint on their cuticle– affects their exploratory and recruitment behaviour within a colony. Painting and anaesthetization did not affect the number of ants that explored an arena. Within a house hunting context, *T. rugatulus* ants that have been painted and anaesthetized showed a similar propensity to take part in recruiting their nestmates (using tandem run or transport) as control ants that had been neither painted nor anaesthetized. We also did not see any differences between unpainted ants and painted ants in how efficient their routes were during these recruitment events. However, when we looked at the numbers of tandem-run and transport events conducted by recruiters (ants who performed at least one tandem run or transport) in each treatment group, the ants that were painted with low temperature anaesthetization were found to perform the task at higher levels than unpainted ants.

What physiological effects could exposure to anaesthetics have on ants that changes their individual behaviour? While the effect of anaesthetization has not been investigated specifically in *T. rugatulus*, researchers have studied this effect on other insect models. These studies have shown that exposure to low temperature may affect Juvenile Hormone titre levels, which have been shown to be correlated with activity levels (Poissonnier et al., 2015; Robinson, 1992). For example, bumblebees spent lesser time foraging on exposure to low temperatures compared to unexposed bees (Wilson et al., 2006). However, in our study, cold anaesthetization had no effect on the exploratory behaviour

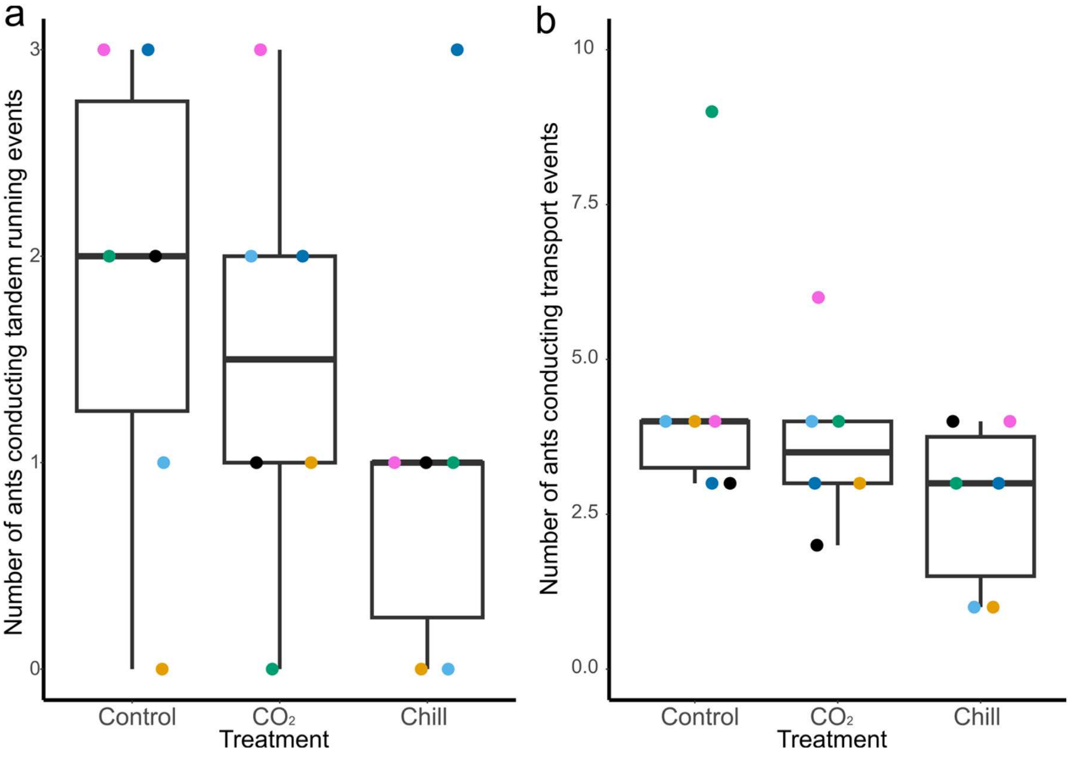

Fig. 4. Box plots showing the average number of ants in each treatment group that perform (a) tandem running and (b) transport events. Each point represents the number of ants that performed at least one TR or transport event, and the colour represents the colony identity. Each box extends between the lower and upper quartiles; the horizontal line within the box indicates the median, and whiskers show the range of the data except for outliers. Outliers are data that lie outside 1.5 times the interquartile range above the upper quartile and below the lower quartile, and are plotted individually (n = 6 colonies).

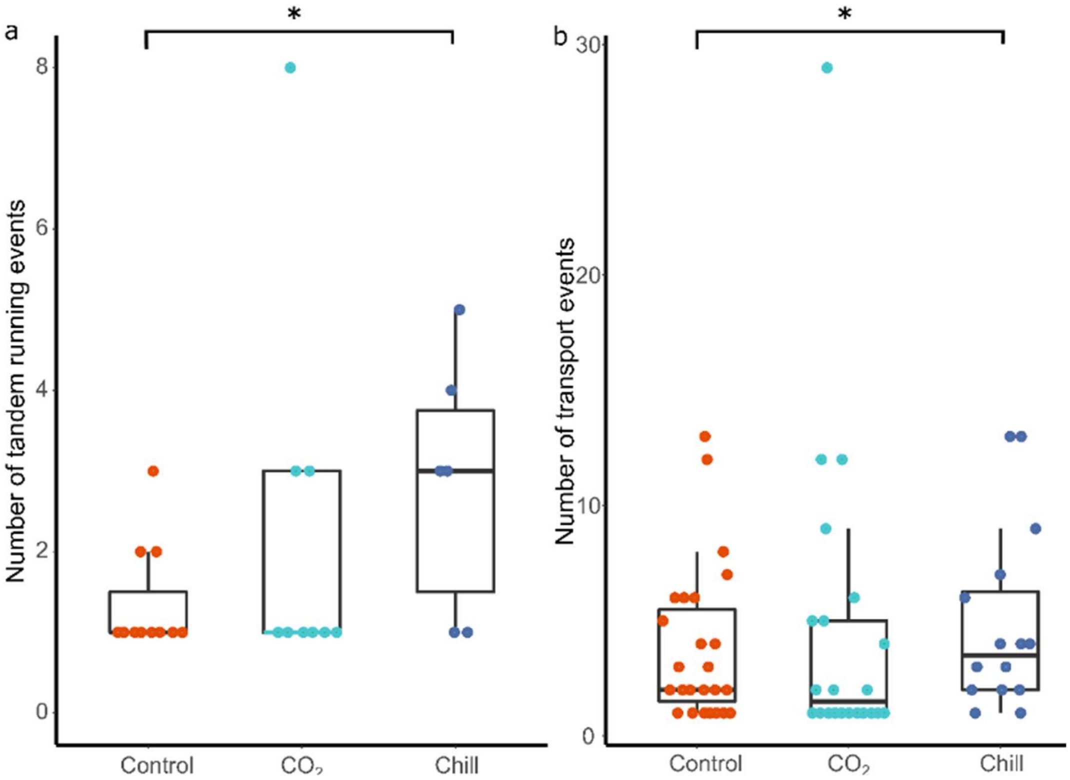

Fig. 5. Boxplots showing the numbers of (a) tandem running and (b) transport events performed by recruiters in the three different treatment groups, red: not painted, cyan: painted on anaesthetizing with CO_2 and blue: painted on anaesthetizing with low temperature. Each point represents the number of tandem-run events or transport events performed by a unique individual. Significant difference between two treatment groups (at p = 0.05) is represented by an asterisk between the relevant bars. Each box extends between the lower and upper quartiles; the horizontal line within the box indicates the median, and whiskers show the range of the data except for outliers. Outliers are data that lie outside 1.5 times the interquartile range above the upper quartile and below the lower quartile, and are plotted individually (n = 6 colonies; number of tandem running events: control = 11, CO_2 = 9, low temperature = 6 ants; number of transport events: control = 27, CO_2 = 22, low temperature = 16).

of the ants. Ants that were anesthetized using cold temperature performed more, not fewer, recruitment acts than control ones did. It is therefore still unknown how low temperature increases recruitment acts in *T. rugatulus*. As for the CO_2 anaesthetization, while many studies have found its detrimental effects on learning, behaviour, and memory of insects, the subjects in these studies are often exposed for much longer than needed (e.g., 15 min ((Lum, 1974) and 2 hr (Medugorac and Lindauer, 1967)) for painting them than ones in our study (3–4 min). Thus, our study suggests that shortening the duration of CO2 anaesthetization could avoid unintended effects on behaviour. We might have found negative effects of CO_2 if the duration of exposure were longer.

In addition to affecting how individuals behave, anaesthetization could also affect how individuals within a colony interact with each other, which could affect the performance of collective tasks. Bumblebees exposed to low temperatures showed lower levels of recruitment – thus, it affected their interaction with nestmates (Wilson et al., 2006). *Formica* ants exposed to low temperatures showed higher aggression against their nest mates (Tanner, 2009). Bumblebee colonies exposed to low temperatures and CO_2 showed different patterns of nestmate aggression compared to control colonies (Poissonnier et al., 2015), which indicates that these anaesthetization processes could be interfering with the nestmate recognition mechanism in these colonies. In contrast to the results obtained by these studies, we found that *Temnothorax* ants were more active and made more recruitment trips after being anaesthetized with low temperatures and were not different from control ants on being anaesthetized with CO_2. Our data suggest that responses to different forms of anaesthetization may be taxon specific, and we need more studies across social insect species to understand these responses.

A few studies have looked at the specific effect of paint marks (without the use of any anaesthetic) on social insect behaviour. For example, in *Polistes versicolor*, wasps with paint applied on their thorax groomed themselves more than wasps where the thorax was touched with a clean brush but no paint was applied (De Souza et al., 2012). Furthermore, lab studies on halictid bees found that different species reacted differently to painting – the solitary *Penopsis toroi* bees were more aggressive to each other on painting, while the semisocial *Corynura cloris* showed more cooperation with each other (Packer, 2005). These results suggest that painting could cause differences in interactions among colony members and impact performance of collective tasks. Social insects primarily interact with each other through tactile and chemical means, and the presence of paint on their bodies could obscure the ability of individuals to sense and communicate with their nestmates (Hölldobler and Wilson, 1990). While our data showed no effect of painting on the individual's participation in the collective task of emigrations, we did not directly measure collective performance, such as emigration speed and nest choice. Future research should investigate if the paint affects collective performance by, for example, painting all colony members and comparing their performance with that of unpainted colonies.

One limitation of our study is that we were able to record only a subset of the entire process. Because we had to manually follow each ant in order to record individual-specific data, when ants aggregated and overlapped with each other, it became extremely difficult to distinguish

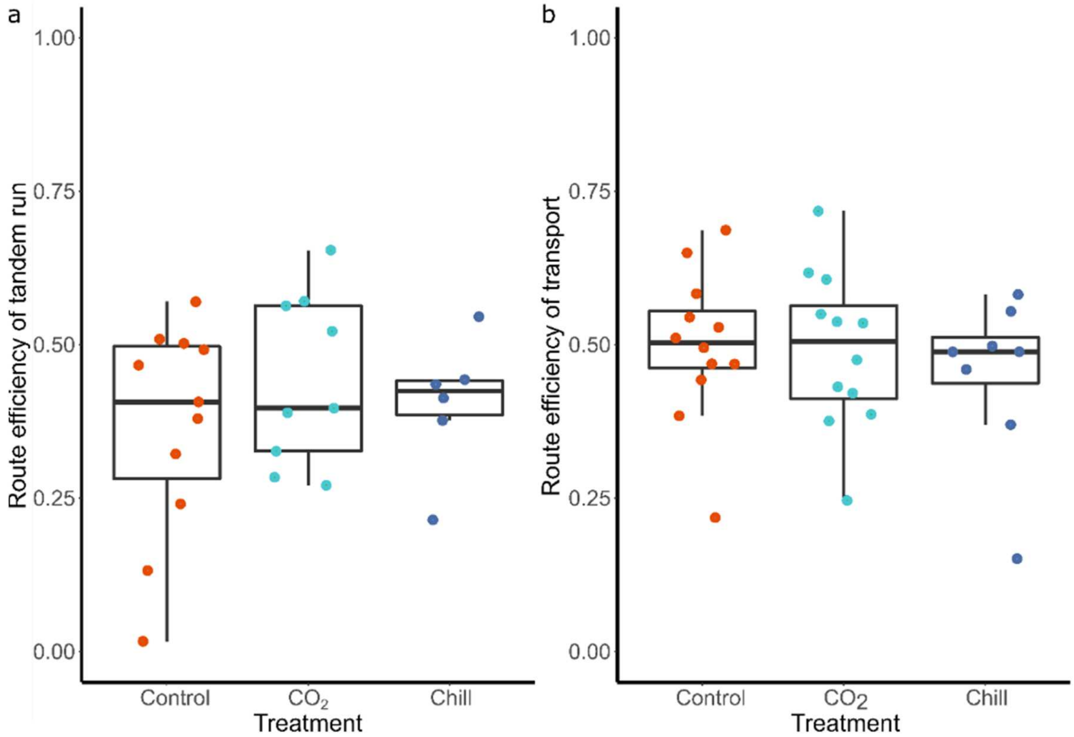

Fig. 6. Boxplots showing the efficiencies of route of (a) tandem running and (b) transport events performed by recruiters in the three different treatment groups, red: not painted, cyan: painted on anaesthetizing with CO_2 and blue: painted on anaesthetizing with low temperature. Each point represents the route efficiency of a tandem run or transport event averaged for each individual (if an individual only performed one event that value is plotted). Each box extends between the lower and upper quartiles; the horizontal line within the box indicates the median, and whiskers show the range of the data (n = 6 colonies; Tandem running – control – 11 ants, CO_2 – 9 ants, low temperature – 6 ants; Carrying – control – 12 ants, CO_2 – 12 ants, low temperature – 8 ants).

between individuals. Therefore, we conservatively used only subsets of scouts and transport recruiters for our analyses. In addition, for the route information of transport recruiters, we manually traced it for each trip so that the amount of data was limited due to the time constraint. Considering that a small subset of *Temnothorax* colony members typically participates in these tasks and starts doing so from the beginning of the event (Dornhaus et al., 2008), we expect that our small dataset, which looks at only the early part of the whole event, is still a good representation of the whole distribution. However, additional data will be required to confirm this point. For example, many animal tracking software applications are available today and have been rapidly progressing (Panadeiro et al., 2021) so that future applications may allow us to track individual ants accurately and efficiently even when these ants are aggregated.

Our study has investigated, for the first time, the effect of painting and anaesthetization on not only individual behaviour, but their participation in a collective task. We found that, in our ant species, *T. rugatulus*, the CO_2 anaesthetization method did not affect activity levels of their exploratory or recruitment behaviours. Thus, this paint-marking method is valid and would be an ideal choice when marking *T. rugatulus* colonies for experiments. Documenting the effect of anaesthetization and painting on behaviour is important because researchers can make careful choices about the methods they use when performing behavioural experiments. By doing so, researchers can avoid using the methods that could affect the behavioural responses they are trying to measure and drawing wrong conclusions in their experiments.

CRediT authorship contribution statement

Supraja Rajagopal: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Methodology, Writing – original draft. **Takao Sasaki**: Conceptualization, Methodology, Resources, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors do not have any competing interests.

Data availability

I have shared the link to my data at the Attach files step.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.beproc.2023.104985.

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