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# Testing optimal defence theory in a social insect: Increased risk is correlated with increased venom investment

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## Abstract

1. Investment in defence may not be uniformly beneficial across an organism's lifespan. Risk, and therefore potential payoff of defence, may change with age and behaviour, but for colony-living organisms, it may also change with colony size and reproductive stage.

2. The acorn ant *Temnothorax longispinosus* uses venom to defend against socially parasitic ants that raid their nests to steal brood. We investigated the idea that *T. longispinosus* adjust their venom allocation in accordance with raid risk. We tested the predictions that *T. longispinosus* ants should produce more venom when raids are most likely to occur and during the parts of the nest reproductive cycle when the potential fitness loss per raid is highest. We also asked whether venom volume varies between nurses and foragers within a colony, which have different potential risk levels, and whether this difference increases with colony size.

3. We found that workers had more venom in the summer, both before and during the period when raids occur, than in the fall when pupae were no longer present in nests. Workers engaging in nursing behaviours had more venom as the pupa to worker ratio in the nest increased, indicating that nurses invest more in venom as the relative number of pupae requiring defence increases. In addition, the difference in venom volume between nurses and foragers grew with colony size.

4. These results provide observational support for the hypothesis that individuals vary their investment in venom over their colony's development in conjunction with the risk of social parasitism.

**KEYWORDS**
ants, chemical defence, social insects, *Temnothorax longispinosus*, trade-offs, venom

## INTRODUCTION

Over the course of an organism's development, resources like space and nutrients limit growth (Emlen, 2001). This can lead to evolutionary trade-offs, as resources are strategically allocated to optimise fitness (Stevens et al., 2000). One important trade-off is between investment in defence versus investment in growth and reproduction. The optimal defence hypothesis (ODH) states that organisms evolve to allocate defence in a way that optimises inclusive fitness (Feeny, 1976;

McKey, 1974; Rhoades, 1979; Stamp, 2003). The ODH was first conceived as an explanation for how defence trade-offs are resolved in plants that produce chemicals to deter herbivores, but it applies to any organism that produces chemicals or uses defensive behaviour to deter threats. Social insects experience strong intra- and inter-specific competitions, have many specialised parasites and predators, and have a wealth of defensive strategies including venoms and noxious secretions (Abbot, 2022), and so we can use this same framework to help explain how defence trade-offs are resolved in ants.

ODH predicts that investment in chemical defences should be allocated in proportion to the risk to the particular tissue and its value to the organism's fitness, and in inverse proportion to the costs of defence (Stamp, 2003). Most studies of ODH have focused on plants and have only considered direct fitness benefits, though a few have considered how plants may alter respective investment in growth, reproduction, and defence to increase their inclusive fitness when clones or closely related individuals are nearby (Gruntman & Novoplansky, 2004; Karban & Shiojiri, 2009). Select studies in animals have investigated how investment in chemical production may change over individual ontogeny in accordance with the diet, behaviours, and movement abilities associated with different life stages (Andrade & Abe, 1999; Brodie Jr & Formanowicz Jr, 1987). In the context of venom-producing social insects, ants within a nest are typically closely related, and so the inclusive fitness of an individual can be affected by the survival of queens, males, workers, larvae, and pupae in their nest (Queller & Strassmann, 1998). Thus, individuals may gain indirect fitness benefits (Brown, 1980) from defending others if participating in defence increases the survival of reproductive kin or of individuals that can rear and defend reproductive kin. Therefore, we do not expect individual ants to invest in venom in direct proportion to the risk they experience individually, but rather in proportion to the risk to their inclusive fitness and the potential benefit of their investment in chemical defence, which depends on the risk to current and future reproductives, the individuals with the potential to rear and defend reproductives and their relatedness to the reproductives.

Investment in venom can be considered in multiple ways, because venomous organisms must produce venom, but also make choices about when to use it and how much to use. In the fire ant, *Solenopsis invicta*, defenders have been found to adjust their venom doses in accordance with potential risk to their fitness, using more venom when protecting reproductive brood than worker-destined brood (Haight, 2018). Haight (2018) leveraged an ant species that has well-studied venom, is relatively large and is known to be aggressive. In venomous ants where we know little about their venom composition and use, and where we are unable to measure venom per sting in the lab, we can still make predictions about how they should vary investment in venom, in terms of production, with colony and ecological conditions. Because venom is metabolically costly to produce (Morgenstern & King, 2013) and the value of venom is likely to vary with individual and colony characteristics, natural selection should act to vary investment in venom, in terms of production, in relation to the net inclusive fitness payoff.

Venom is used for a variety of purposes in ants, including defence, predation, as an herbicide, as a disinfectant, and for communication (Touchard et al., 2016). We know little about venom composition and use in the venom-producing acorn ant *Temnothorax longispinosus*. *T. longispinosus* are common in the Northeastern USA, nesting inside small cavities including hollowed out acorns, hickory nuts and twigs. Their diet and habitat preclude predation and herbicide as uses of venom. Disinfection is a possible use of venom that has not been studied in this species. Venom is used as a trail pheromone in some ants from the same subfamily (Piek, 1986), but workers

**TABLE 1**    List of predictions that follow from optimal defence theory.

| Prediction |
| --- |
| 1. Workers will have increased venom during times of year when parasitic raids are most likely to occur, and decreased venom when raids are unlikely |
| 2. Workers will have increased venom when potential losses during a raid are higher, i.e., when the ratio of pupae to workers is higher |
| 3. Foragers will have more venom than nurses because they have a higher risk of encountering opponents |
| 4. The difference in venom volume between foragers and nurses will increase with colony size because the specialisation of behavioural castes often increases with colony size, and so the difference in potential risk between foragers and nurses will also increase with colony size |

of *T. longispinosus* are solitary foragers (Modlmeier & Foitzik, 2011), so trail pheromones are likely only used during nest relocation. However, *T. longispinosus* are known to use their venom when defending against raids by *T. americanus*, an obligate social parasite for which they are the preferred host (Foitzik et al., 2009). *Temnothorax americanus* raid *T. longispinosus* nests to steal brood, timing their raids to coincide with the period of time when pupae are available in the nest, which is estimated to be ~50 consecutive days per year in Ithaca, NY (Foitzik & Herbers, 2001b; Miller, 2021). Because the chance of experiencing raids, and the potential risk to fitness, vary with seasonality and nest contents, this is a good system in which to consider how investment in venom, in terms of the amount of venom workers have, should vary with the potential payoff of defence.

Because the chance of a *T. longispinosus* nest experiencing a raid varies seasonally, the chance of engaging in defence and the potential payoff of venom production should also vary seasonally (Table 1, prediction 1). The likelihood of a *T. longispinosus* nest being successfully raided in a year has been estimated to be about 50% (Foitzik & Herbers, 2001b). *T. longispinosus* are facultatively polygynous, meaning that one colony can have multiple laying queens. Queens within a nesting unit share about half of their alleles with each other, and queens seem to be singly mated, with the average worker-worker relatedness within a nest typically above or around 0.5, even when there are many queens (Foitzik & Herbers, 2001a; Herbers & Stuart, 1996). *T. longispinosus* are also facultatively polydomous, meaning that one colony can be spread among multiple nests. However, because there is no spatial genetic structure between nests, the nest has been inferred to be the functional unit of selection (Herbers & Stuart, 1996), and since raids are experienced on a nest-by-nest basis, ants should evolve to optimise their investment in defence on a nest level. If a raid is initiated by a parasite colony, the number of stolen brood items increases with the ratio of brood to workers within the host colony (Miller, 2020). Because pupae are extremely valuable to the inclusive fitness of individuals, and the potential payoff of venom increases with the brood to worker ratio, individual workers should invest more in venom as the pupa to worker ratio increases (Table 1, prediction 2).

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Most ant societies, including *T. longispinosus*, have a division of labour among the workers, where the tasks they engage in depend on their behavioural caste (Herbers & Cunningham, 1983; Hölldobler & Wilson, 1990). Because the chances of an individual encountering opponents vary with task, and the tasks that individuals perform depend on their behavioural caste, the optimal investment in defence for an individual worker should vary with behavioural caste (Table 1, prediction 3). However, the patterns of division of labour within a colony may vary with colony characteristics (Traniello & Rosengaus, 1997). In some social insect species, the specialisation of behavioural castes, in terms of the behaviours they perform, has been found to increase with an increase in colony size, especially for defensive tasks (Amador-Vargas et al., 2015; Holbrook et al., 2011; Pacala et al., 1996). If specialisation of behavioural castes increases with colony size, the difference in potential risk between foragers and nurses should increase with colony size, and, therefore, the difference between the amount of venom a forager produces and the amount a nurse produces should increase with colony size (Table 1, prediction 4).

In summary, we investigated venom volume in *T. longispinosus* workers in relationship to colony phenology and seasonality, and contextualised results with information on the timing of parasitic raids. We also explored alternative explanations for the patterns in our data. In addition, we investigated how venom volume differs between workers that participate in foraging behaviours and nurse workers that stay in the nest to tend the brood, and how differences in venom between behavioural castes interact with the number of workers and the pupa to worker ratio in the nest.

## METHODS

We collected 172 acorns and hickory nuts containing *T. longispinosus* nests on Cornell Botanic Garden land in Ithaca, NY, USA, between May and October, 2021. Each nest was stored for a maximum of 18 days in the fridge before we censused it to count all live dealate queens, alate queens, workers, males, pupae, larvae and eggs. Each group was transferred to the foraging arena of a $10 \times 10$ cm container with a Plaster of Paris floor and an artificial nest, which consisted of a piece of balsa wood with a 2.54 cm diameter hole sandwiched between $5.1 \times 7.6$ cm microscope slides, with a small tunnel cut in the wood to allow the ants to enter and exit the nest (Sasaki et al., 2021). Each nest had a red filter on top to limit light (Rosco Roscolux 27: Medium Red).

Nests were kept in the lab for between 2 and 8 days to allow them to move all their larvae and pupae into the nest and reorganise. We fed ants a Bhatkar diet (Bhatkar & Whitcomb, 1970) and cricket legs by putting a small petri dish with food in the foraging arena. Once ants had successfully reorganised inside the artificial nest, we observed workers at a single point in time in order to pick out nurses and foragers. Only workers that were outside the nest in the foraging arena were considered foragers. Only workers that were actively tending to brood were considered nurses. Not all workers were classified into a behavioural group. We removed individuals from each nest into Eppendorf tubes, separated by behavioural group, and froze them.

We dissected individuals under an Olympus SZX9 dissecting scope at $20\times$ magnification. If there were more than 20 ants in a behavioural cohort from a single nest, we stopped dissecting after measuring 20 venom sacs. Two pictures of each ant were taken using a microscope-mounted digital camera (Olympus UTV0.63XC): one of the side profile and one of the venom reservoir. Using the photos, we measured Weber's Length (Weber, 1938), a standard measurement used to control for body size in ants, and the length (*L*) and width (*W*) of each venom reservoir (Figure 1), using the camera software (Olympus cellSens Standard 1.17). In total, we successfully dissected 2055 workers (1394 nurses and 661 foragers) from 155 nests. We dissected many more nurses than foragers because the number of workers engaging in foraging behaviours was much lower than the number engaging in brood care during the behavioural observation period. We do not have worker measurements for all nests collected because some nests had few or no workers. Following Haight (2012), we approximated the volume of venom in the venom reservoir for each dissected individual as a prolate spheroid using the equation: Venom volume $\approx \frac{\pi}{6} \times \left( L \times W^2 \right)$.

## Statistical methods

All statistical tests were done in R studio (R version 4.1.3.) (R Core Team, 2022). The complete code, statistical output, and raw data required to replicate all analyses are available (DOI: 10.5281/zenodo. 10059349).

Linear mixed-effects models were built using the lme function from the nlme package (Pinheiro et al., 2022).

We calculated an $R^2$ estimate using the rsq.lmm function from the rsq package (Zhang, 2022).

## Nest census

To determine whether the number of workers in a nest significantly increased over the course of the season, we built a linear mixed-effects model with the number of workers as the dependent variable, colony as a random intercept and the date of collection as a fixed effect ($n = 172$ nests). The number of nests included in the nest census data is larger than the number of nests for which we have venom volume measurements, because some nests contained few or no workers.

## Venom volume

We scaled adult count for each nest by calculating a Z-score (subtracting the mean adult count for all nests and dividing by the standard deviation) using the scale function. To determine how venom volume
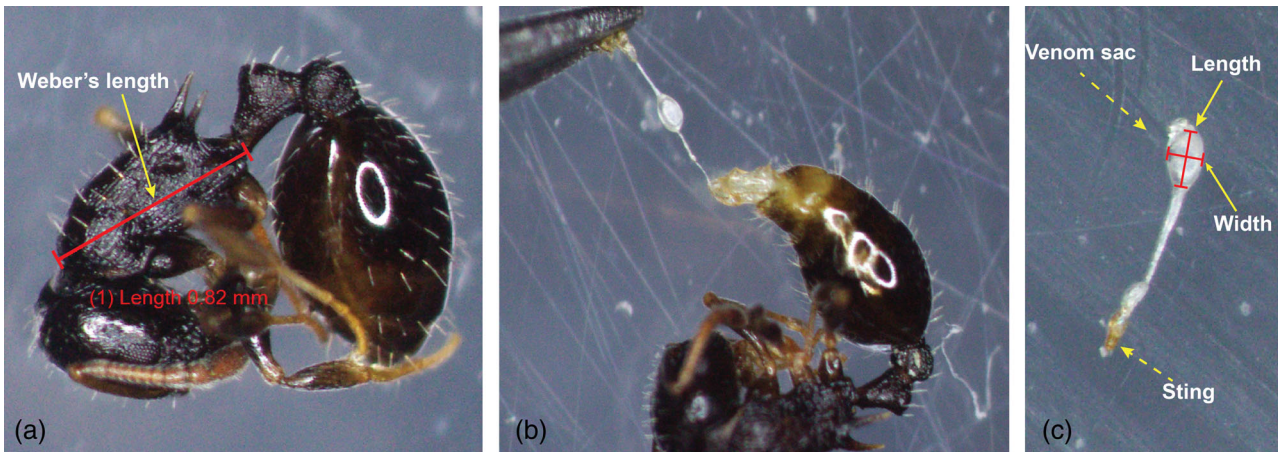
**FIGURE 1** Photographs of *Temnothorax longispinosus* ants and venom sacs taken with a microscope camera. (a) Side profile of ant with Weber's length indicated, which we used to control for body size. (b) Worker during dissection, with venom sac partially removed. (c) Dissected venom sac with sting connected and length and width indicated.

for workers varied with colony characteristics and worker behaviour, we built a linear mixed-effects model with venom volume as the dependent variable and colony as a random intercept. As fixed effects, we included Weber's length[3], collection day, whether the individual was callow (a recently emerged worker with less melanisation), the presence of a dealate queen, whether the worker was a nurse or a forager, the pupa-to-worker ratio, the number of workers, an interaction effect between the number of workers and whether the worker was a nurse or a forager, and an interaction effect between the pupa-to-worker ratio and whether the worker was a nurse or a forager. We included Weber's length[3] to control for a pattern where larger ants have more venom as a result of their body size and cubed Weber's length because it is a one-dimensional measurement, whereas venom is three dimensional. We included collection day as a fixed effect because the chance of raids by *T. americanus* vary seasonally. We included the presence of a dealate queen because the presence of reproductive queen(s) is associated with pheromones that can influence physiology and behaviour in many social insect species (Free, 1987; Holman et al., 2010; Matsuura et al., 2010; Vander Meer et al., 2019), including *T. longispinosus* (Brunner et al., 2011). We used the pupa to worker ratio in the nest, instead of the raw number of pupae, as a fixed effect because the ratio represents the number of individuals that cannot behaviourally defend themselves divided by the number of individuals able to participate in defence. We removed the number of alate queens and the larva to worker ratio from the model during the simplification process because they were not statistically significant predictors of worker venom volume and their removal decreased the AIC score for the model.

Both interaction effects were included to investigate differences between nurses and foragers (hereafter referred to as the "behavioural group" of the worker). Most ant societies have an age-related division of labour among the worker caste, where aging is associated with changes in the tasks they perform (Hölldobler & Wilson, 1990). Patterns of division of labour in social insects are species-specific and

may depend on colony size (Traniello & Rosengaus, 1997), but most species have nurses, which spend time tending to brood within the nest, and foragers, which spend time collecting food outside the nest. We included an interaction between number of workers and behavioural group in order to test the prediction that the difference in venom volume between foragers and nurses would increase with colony size. We included an interaction between pupa to worker ratio and behavioural group because nurses spend more time inside the nest and interacting with nest contents than foragers, and so we wanted to investigate whether patterns of investment based on nest contents would differ between behavioural groups.

## RESULTS

### Nest census

Workers and larvae were present in almost every nest throughout the collection season, beginning at our first collection date in May. We first found pupae in nests on June 14, and most nests we collected contained pupae from then until mid-August. By September, the number of pupae dropped to zero in most nests. Of the 40 nests we collected in September and October, only one contained any pupae. The number of workers in a colony significantly increased as the date of collection progressed from May to October (lm, df = 170, $t$ = 7.4, $p$ < 0.001, Figure 2).

### Venom volume

The statistical results for worker venom volume are summarised in Table 2. The mean venom volume for workers was 1.2 ± 0.56 nL. We collected an average of 3.9 nests per day on 40 unique collection days.
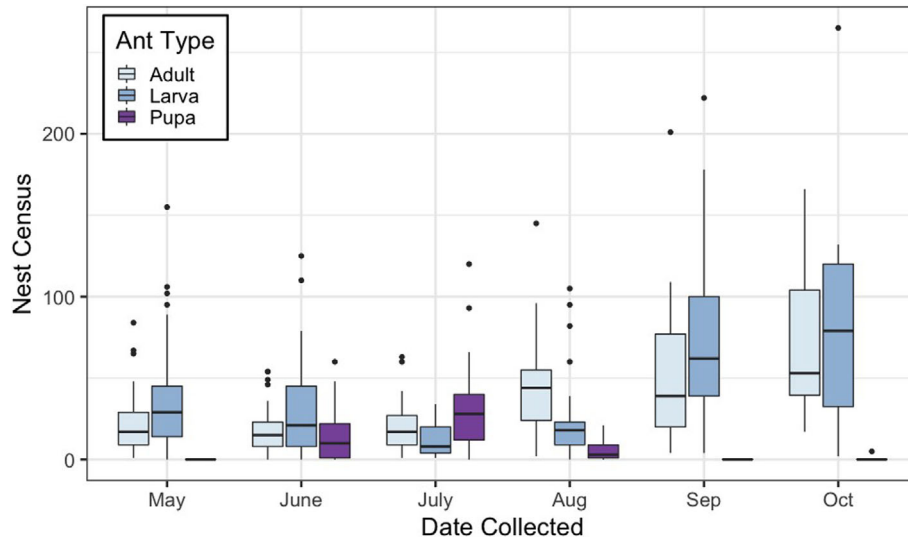
**FIGURE 2** Count of workers, pupae and larvae in all collected nests by date of nest collection. Date of nest collection is binned by month ($n = 172$ nests).

**TABLE 2** Results from linear mixed-effects model predicting venom volume for workers.

| Fixed effects | Est ± SE | t | p-value |
| --- | --- | --- | --- |
| Intercept | 0.88 ± 0.08 | 10.5 | <0.001 |
| Weber's length$^3$ | 1.57 ± 0.14 | 11.21 | <0.001 |
| Behavioural group (nurse) | −0.27 ± 0.03 | −9.32 | <0.001 |
| Day of nest collection | −0.002 ± 0.0004 | −4.74 | <0.001 |
| Presence of dealate queen(s) | −0.08 ± 0.05 | −1.80 | 0.07 |
| Callowness | −0.57 ± 0.12 | −4.54 | <0.001 |
| Scaled number of workers | 0.05 ± 0.02 | 2.05 | 0.04 |
| Pupa to worker ratio | −0.02 ± 0.03 | −0.067 | 0.5 |
| Behavioural group × scaled number of workers | −0.04 ± 0.02 | −2.46 | 0.01 |
|    Scaled number of workers (nurse) | 0.005 ± 0.02 | 0.24 | 0.80 |
|    Scaled number of workers (forager) | 0.05 ± 0.02 | 2.05 | 0.04 |
| Behavioural group × pupa to worker ratio | 0.13 ± 0.03 | 4.07 | <0.001 |
|    Pupa to worker ratio (nurse) | 0.10 ± 0.02 | 4.40 | <0.001 |
|    Pupa to worker ratio (forager) | −0.02 ± 0.03 | −0.67 | 0.50 |

*Note*: Nest identity was included as a random intercept. $R^2 = 0.31$.

Worker venom significantly decreased over the study season from May to November (Figure 3). Venom volume significantly increased with Weber's length$^3$. As predicted, nurse workers had significantly less venom than foragers (Figure 4a), and callow workers had significantly less venom than workers that were fully melanised (Figure 4b). Workers in nests containing at least one dealate queen had slightly less venom than those in nests without a dealate queen (Figure 4c), though this difference was not statistically significant.

There were significant interaction effects between behavioural group and number of workers in the nest, and between behavioural group and the pupa to worker ratio in the nest, in predicting venom volume. The slope for venom volume and number of workers were statistically different between foragers and nurses (Figure 5). In the linear mixed-effects model, nurses and foragers from nests with more workers had more venom than those from nests with fewer workers, but this was only statistically significant for foragers.

The effect of pupa to worker ratio on venom volume was statistically different between foragers and nurses (Figure 6); nurses from nests with a higher pupa to worker ratio had significantly more venom than nurses from nests with a lower pupa to worker ratio. There was no statistical difference in venom volume of foragers depending on the pupa to worker ratio.
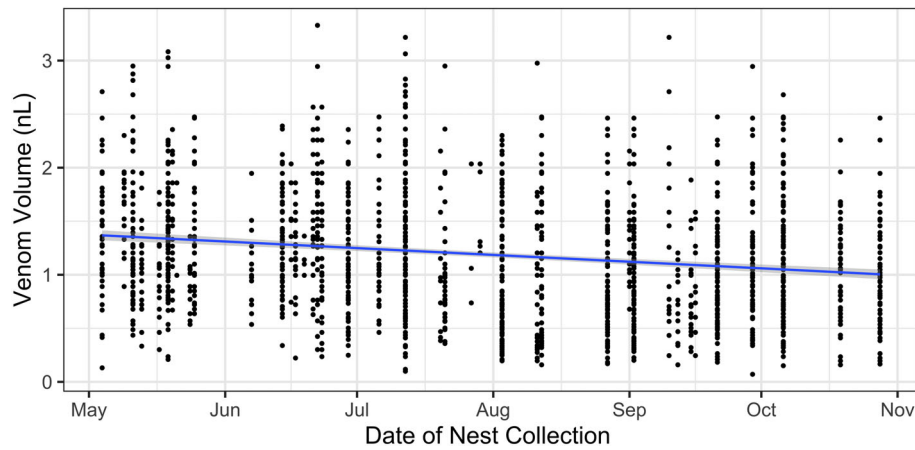
**FIGURE 3**  Venom volume, in nL, of workers by the date on which their nest was collected. lm ± se ($n$ = 2055 workers from 155 nests).
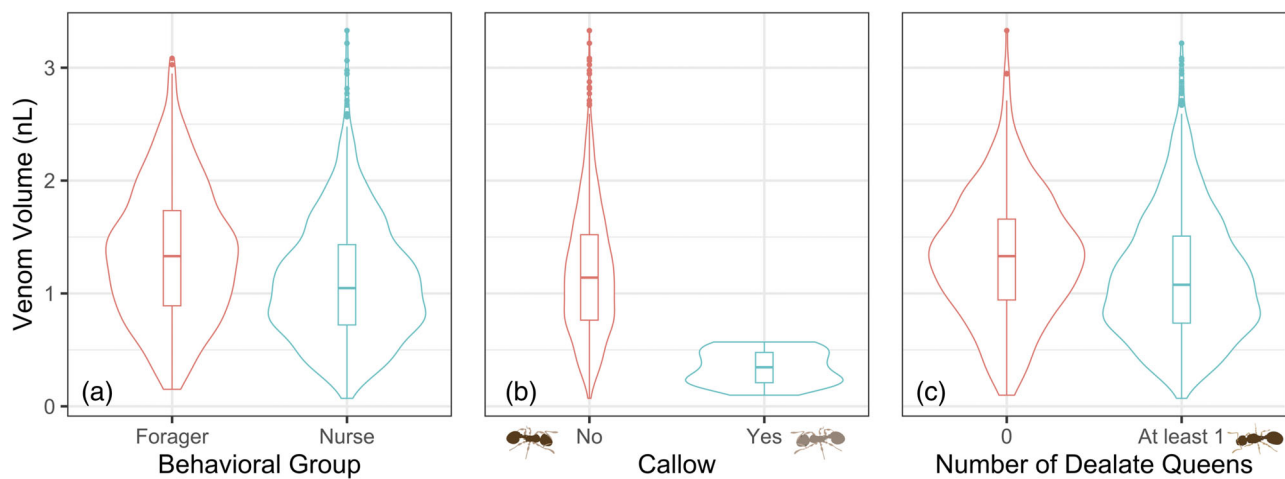


**FIGURE 4**  Venom volume, in nL, of workers by (a) behavioural group: whether the worker was engaged in foraging or nursing behaviours during the behavioural observation (661 foragers from 142 nests and 1394 nurses from 153 nests), (b) Callowness: whether or not the worker was callow, meaning they had a light-coloured exoskeleton characteristic of ants that have hatched in the last couple days and have not yet fully melanised (2039 non-callow ants from 155 nests and 16 callow ants from eight nests), (c) presence of one or more dealate queens in nest: whether or not there was at least one wingless queen inside the nest (438 workers from 43 nests without a dealate queen, 1617 workers from 112 nests with at least one dealate queen). lm ± se.

## DISCUSSION

In this study, we aimed to determine how worker venom volume in *T. longispinosus* ants varies according to apparent risk of experiencing a raid, as indexed by nest characteristics, seasonality and behaviour while controlling for ant body size. All individuals within a nest experience high risk during the brief annual period where pupae are present in the nest and, therefore, raiding events occur. We estimate that raids were most likely to occur between mid-June and mid-August during this study, when most nests we collected contained pupae. Nests with higher pupa to worker ratios are no more likely to be raided (Miller, 2021). However, when the number of workers is kept constant, a higher pupa to worker ratio is associated with a higher potential loss, in terms of brood items, when a raid occurs (Miller, 2020). Pupae provide an important value to the inclusive

fitness of individuals within a nest, because they do not need to be fed and will become adult workers that can rear and defend reproductives. Since pupae are unable to behaviourally defend themselves, as the number of pupae relative to workers increases, the proportion of individuals in the nest that are able to behaviourally participate in defence decreases. Thus, we would expect those that are able to behaviourally defend to invest more in venom. As predicted (prediction 2, Figure 6), nurses in nests with higher pupa to worker ratios had more venom than those in nests with lower pupa to worker ratios. However, we were surprised to find that this pattern was specific to nurses and did not occur in foragers.

Some species of ants have been found to use glandular secretions to sanitise their nest, materials they collect, their nestmates or brood (Brütsch et al., 2017; Pull et al., 2018; Tranter et al., 2014). For example, workers of the ant species *Lasius neglectus* have been found to
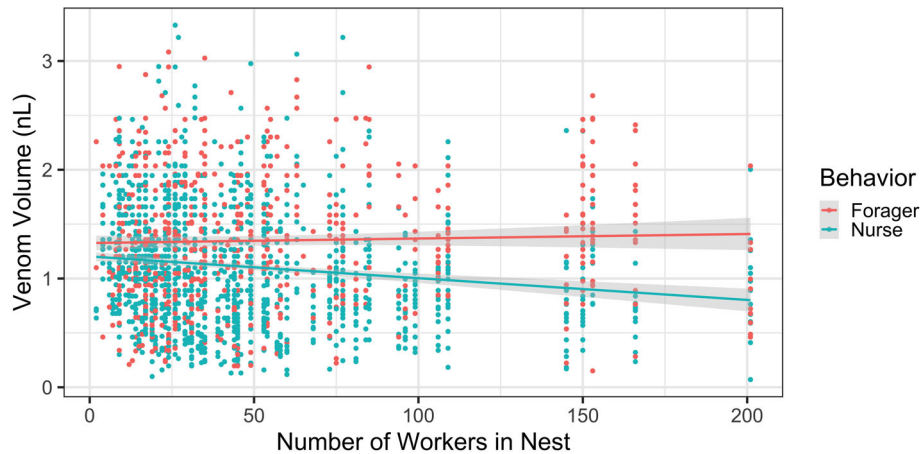
**FIGURE 5** Venom volume, in nL, of workers by the number of workers present in the nest upon collection. lm ± se. Red represents dissected foragers. Blue represents dissected nurses ($n = 1394$ nurses and 661 foragers).
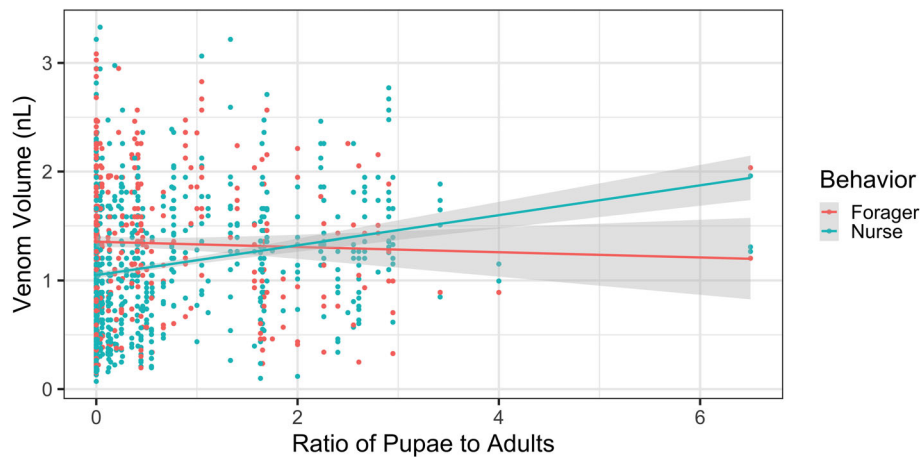


**FIGURE 6** Venom volume, in nL, for workers by the pupa to worker ratio in the nest upon collection. lm ± se. Red represents dissected foragers. Blue represents dissected nurses ($n = 1394$ nurses and 661 foragers).

use poison from their acidopores to groom fungus-contaminated pupae, which inhibits fungal growth on the pupae (Tragust et al., 2013). *Lasius neglectus* differ from *T. longispinosus* in that they have an acidopore as opposed to a sting. To our knowledge, no ants with stings, like *T. longispinosus*, have been experimentally found to disinfect brood with their venom. However, this could be because it has not been well studied. Fire ants (*Solenopsis invicta*), which are in the same subfamily as *T. longispinosus* (Myrmicinae), have been observed to raise their abdomens and air-disperse venom from their stings onto their brood through "gaster flagging" behaviour, which is hypothesized to be for disinfection (Obin & Vander Meer, 1985). The use of venom by ants as a disinfectant of brood warrants more research. If *T. longispinosus* nurses use their venom to disinfect pupae, it could explain the pattern we observed where nurses, but not foragers, have more venom as the pupa to adult ratio increases. In this case, nurses would need to produce more venom as the pupa to worker ratio increases, because each nurse would be responsible for sanitising more pupae.

Measurements of venom volume require destructive sampling and indicate the amount of venom individuals have at a single snapshot in time. Consequently, we are unable to distinguish differences in production with differences in use or individual age. An increase in venom for nurses as the pupa to adult ratio increases could be explained by nurses using less venom as the pupa to adult ratio increases; however, we cannot think of a compelling reason why this would be. Another possibility is that nurses in nests with high pupa-to-worker ratios are older than those in nests with low pupa-to-worker ratios, and therefore have had more time to produce venom. We do not know the ages of the individuals we dissected; however, if this were the case, we would expect the same pattern to hold for foragers.

Alternatively, the factors that determine investment in venom for individual ants may change as they age and switch behavioural groups. Since nurses spend the majority of their time inside the nest, their investment in venom could be more heavily influenced by nest characteristics, which they are interacting with constantly. In contrast,

foragers are interacting with the broader world, and their investment in venom may be more heavily influenced by cues they receive while foraging. As predicted (prediction 3, Figure 4a), we did find that foragers, on average, had more venom than nurses, which makes sense because they tend to engage in more dangerous tasks than nurses.

We also found a significant interaction effect between the number of workers in a nest and the behavioural group of a worker in predicting venom volume, with the amount of venom for a forager significantly increasing with number of workers (Figure 5). Thus, the difference between the predicted amount of venom for a forager and a nurse increased as the number of adult workers in a nest increased. This pattern supports the prediction (prediction 4) that workers have increased task specialisation as nest size increases, a pattern that has been found for defensive tasks in other ant species (Amador-Vargas et al., 2015). If workers perform tasks that are more specialised as colony size increases, we expect that nurses and foragers both participate in defence in a small colony, and that nurses should be less likely to participate in defence as the colony size increases. Therefore, the risk nurses' and foragers' experience should be more similar in a small colony than in a large colony. Consequently, the amount of venom nurses and foragers produce should be more similar in a small colony than in a large colony, which we found in our study.

We do not know the rate at which individuals are using venom, and this pattern could also be explained by differential use if foragers tend to use less venom as the number of workers in the nest increases, and nurses use the same amount of venom regardless of the number of workers in the nest. Because we do not know the age of individuals we dissected, it is also possible that the average age of a forager increases with colony size, and older individuals have more venom.

On average, workers had less venom as the season progressed between May and October (Figure 3). During the 2021 field season, we began to see pupae in *T. longispinosus* nests in mid-June, and the number of pupae peaked in July. The majority of nests we collected did not contain any pupae by mid-August, indicating that all pupae for the season had hatched. Pupae are more valuable than larvae because they do not need to be fed and are destined to hatch during the current summer. It can take larvae almost a year to pupate, and so while larger, mature larvae are sometimes stolen opportunistically during raids, larvae are not uniformly valuable. Most larvae present in nests after mid-August are not likely to be stolen this year. They will overwinter as larvae and pupate the following year. Therefore, after the pupae for the year have hatched, the risk of experiencing a raid should drop, as well as the potential fitness loss during a raid (Miller, 2020), and so we would expect *T. longispinosus* to invest less in venom. As predicted (prediction 1), we observed a seasonal decrease in venom that corresponded with the decrease in potential for raid events, which are timed to coincide with the brief period of time when pupae are present in *T. longispinosus* nests (Foitzik & Herbers, 2001b). However, we did not collect data on raid occurrences and so cannot directly connect the decrease in venom volume at the end of the season with the risk of experiencing a raid. In addition, raiding risk does not explain why workers had high levels of venom in May and early

June, before raids were likely to occur. The pattern of venom synthesis over the ontogeny of individuals has not been studied in *T. longispinosus*, but studies from other species (Haight, 2012), along with our data showing that callow workers have less venom than fully melanised workers, indicate that it can take time for ants to synthesise venom. Even though nests are not likely to experience raids before brood pupates in mid-summer, worker venom synthesis may need to begin in advance to produce enough venom before raiding season commences. If worker venom production decreases after raiding season ends, more resources could be allocated to preparing for the winter. It is also possible that *T. longispinosus* workers need venom in the early summer for other reasons, which result in high venom investment. Previous work has shown that *T. longispinosus* spread themselves among multiple nest sites during the spring, and maintain this organisation throughout the raiding season. Then, when winter is approaching, they condense to overwinter in larger groups (Herbers & Tucker, 1986). In the spring and early summer, workers may experience risk as they move and transport larvae between nests, which might explain why workers seem to have similar relative amounts of venom in the early summer as they do in mid-summer, once raiding season has commenced. Nest relocation could also require venom for trail pheromones if scouts use venom to lay trails to new potential nest sites and coordinate emigration.

This pattern could alternatively be explained by differential use if ants use more venom as the season progresses. However, we cannot think of a reason why this would be with our current knowledge of how *T. longispinosus* are using venom. Age could also contribute to this pattern, if the average age of individuals is highest in May and decreases throughout the season; however, age seems unlikely to be driving this pattern since the average age of individuals in the nest should increase between May and July, before any adults have hatched yet, and between September and November, after all pupae for the year have hatched.

Workers in queenless nests had slightly more venom than those in nests with one or more dealate queens (Figure 4c), though this difference was not statistically significant. Since *T. longispinosus* queens have pheromones that can alter worker physiology and behaviour (Brunner et al., 2011), and the removal of reproductive queens increases ovary development and worker-worker aggression (Konrad et al., 2012), we included this effect in order to control for possible differences between the nests with and without a dealate queen. However, we do not know when or how nests in our study became queenless, and, therefore, a queen removal experiment would give a better sense of how queen status affects worker physiology with respect to venom production.

Our findings support the hypothesis that venom volume in *T. longispinosus* workers varies in accordance with the potential loss of pupae given that a raid occurs, and in accordance with the predicted individual risk of workers based on their behavioural group. Our findings also provide correlative evidence that venom investment decreases with seasonal raid risk. In addition, we found that the difference in venom volume between nurses and foragers increased with colony size, providing evidence that *T. longispinosus* workers may

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become more specialised in the tasks they perform as colony size increases. These results provide support for the idea that because chemical defence is costly, organisms should optimise their investment according to their risk of needing to defend and the potential payoff of defending given that it is required. Together, this provides observational evidence in support of optimal defence theory in a colony-living animal, though alternative explanations for the patterns we observed cannot be ruled out since little is known about *T. longispinosus* venom composition, investment, and use. Uses are not mutually exclusive, and so *T. longispinosus* could use venom for other purposes in addition to defence, such as disinfecting brood. This work could be followed up on experimentally, by altering nest contents or raid risk for host nests to see if hosts alter their venom volume in response.

## AUTHOR CONTRIBUTIONS

**Phoebe A. Koenig:** Conceptualization; investigation; funding acquisition; writing – original draft; formal analysis; project administration; data curation; resources; writing – review and editing; methodology. **Corrie S. Moreau:** Writing – review and editing; supervision; resources.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Temnos2021 at https://doi.org/10.5281/zenodo.10059349.

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