Effects of nutritional manipulation on intranidal social behaviour in a small carpenter bee

Jesse L. Huisken , Sandra M. Rehan*

Department of Biology, York University, Toronto, ON, Canada

Understanding how subordinate altruistic roles arise among genetically similar individuals is critical to understanding the evolution of animal societies. In particular, maternal manipulation of offspring resources is an understudied process by which mothers may produce cooperative phenotypes among offspring. Mothers of the small carpenter bee *Ceratina calcarata* frequently produce a smaller under-provisioned daughter, known as the dwarf eldest daughter (DED) in the first cell of their nests. This DED is more subordinate and more easily coerced into helping siblings, aiding mothers in guarding and supplemental foraging and feeding of adult siblings. Colonies of these bees thus offer a unique opportunity to study the indirect effect of nutritional manipulation on behaviour, in particular mutual tolerance. We hypothesized that aggression would be greatest in the presence of DEDs, as they are actively coerced by their mothers to forage and feed their siblings. Here we experimentally added nutrition to the first cells of *C. calcarata* observation nests to test the effect of nutritional manipulation on colony intranidal behaviour. Using infrared cameras, control ($N = 13$) and nutritionally supplemented nests ($N = 13$) were observed for a total of 607.5 h. Behaviours were scored and categorized. Although aggression was unaffected, avoidant behaviour was greater in nutritionally supplemented nests. This study is the first experiment examining the effect of nutritional manipulation on intranidal behaviour in a stem nesting bee. Our results indicate that maternal manipulation and nutritional deprivation by mothers is critical to the production of mutual tolerance and division of labour.
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Parental manipulation may have profound influences on offspring development, physiology and behaviour (Reinhold, 2002; Wolf & Wade, 2009). The ability of parents to produce variable offspring suggests that parental manipulation may be one pathway by which animal societies have evolved (Alexander, 1974; Michener & Brothers, 1974; Craig, 1979). In species exhibiting extended parental care, offspring frequently benefit from food resources and protection from predators and parasites, and may be subject to differential allocation of these resources (Charnov, 1978; Queller, 1996). Manipulation may serve to restrict opportunities for some offspring, compelling them to seek indirect fitness benefits by helping siblings (Alexander, 1974; Crespi & Ragsdale, 2000). Diverse taxa have converged on parental manipulation as a method of producing variation in offspring, including several species of cooperatively nesting birds, eusocial mammals and many bees and wasps (Alexander et al., 1991; Briga et al., 2012; Clarke, 1984; Gadagkar et al., 1991; Hogendoorn et al., 2001;

Koenig & Dickinson, 2004; Packer & K02erer, 1985). Nutrition plays a role in all categories of social behaviour within Hymenoptera, from the manipulation of offspring size in primitively eusocial colonies to the regulation of task specialization in advanced eusocial species (Kukuk & May, 1991; Sullivan & Strassmann, 1984; Wheeler, 1991). Within mass-provisioning facultatively eusocial bees, production of smaller-sized offspring is thought to facilitate behavioural domination and coercion of helpers (Hogendoorn & Velthuis, 1999; Michener & Bennett, 1977; Rehan & Richards, 2013; Smith et al., 2009). In primitively eusocial bees and wasps, adult body size may influence rank within a colony (Hunt & Nalepa, 1994; Michener, 1974; Packer & Knerer, 1985; Tepedino & Torchio, 1982). Within the eusocial Hymenoptera, with fully developed castes and reproductive division of labour, nutritional manipulation determines reproductive status and even task specialization (Trible & Kronauer, 2017; Toth & Robinson, 2005; Wheeler, 1991). Nutritional manipulation offers a proximate mechanism by which parents may produce different social roles in their offspring, and thus frequently plays a determining role in overall colony functioning in social insects (Gadagkar et al., 1991; Hunt & Nalepa, 1994).

The influence of nutrition on development and behaviour is well documented and has been subjected to experimental study within several eusocial insect species (Eickwort, 1969; Gadagkar et al., 1991; Mead et al., 1994; Rossi & Hunt, 1997). The importance of nutrition in establishing cooperation and mutual tolerance in simple social systems, such as those found in the *Ceratina* small carpenter bees, has received less attention (Lawson et al., 2021; Rehan, 2020). Understanding how nutrition influences such simple social systems may reveal how cooperation emerged from solitary ancestry, setting the stage for the evolution of eusociality in Hymenoptera. The eastern North American small carpenter bee, *Ceratina calcarata,* engages in extended parental care of offspring, continuing provisioning offspring after they have fully matured (Rehan & Richards, 2010). The colony cycle of *C. calcarata* progresses from dispersal and mating in early spring, when females establish nests by excavating a linear cavity in the central pith of a dead plant stem (Fig. 1) to mass provisioning and egg laying in early June (Rehan & Richards, 2010, 2013). After offspring have consumed their provisions and developed into adults, they remain in the nest and their mothers feed them during a second period from late July into mid-September (Rehan & Richards, 2010). During this second foraging period, mothers are frequently aided by a smaller daughter ('dwarf eldest daughter', DED), who emerges from the first provisioned cell (Mikát et al., 2017; Rehan & Richards, 2010). As is found in other mass-provisioning bees, adult body size is correlated with larval provisions (Boomsma & Eickwort, 1993; Brand & Chapuisat, 2012; Packer & Knerer, 1985; Quezada-Euán et al., 2011). In *C. calcarata*, the small size of this daughter is the direct result of reduced quality and quantity of larval provisions in the first cell of the nest (Lawson et al., 2016, 2017). Unlike her siblings, this DED exhibits altruistic care and does not survive overwintering to disperse, mate and found her own nest (Rehan & Richards, 2010). Such helping at the nest provides inclusive fitness benefits to DEDs and significantly improves the survivorship of siblings and thus the mother's direct fitness (Shell & Rehan, 2018). These smaller DED offspring are found in several species of the *Ceratina* genus, where they tend to be aggressed by mothers to encourage feeding of nestmates (Sakagami & Maeta, 1977, 1984). Subordinate DEDs have been experimentally produced by manipulating pollen provision quality and quantity within *C. calcarata* (Lawson et al., 2017).

The social status of DEDs has mainly been deduced by assaying individual encounters in forced association circle tube assays (Arsenault et al., 2018; Rehan & Richards, 2013). This technique involves pairing bees in a single length of plastic tube connected at either end (Packer, 2006; Robinson & Page, 1989). Studies of intranidal behaviour within primitively eusocial bees have determined nestmate recognition and behaviours associated with caste specialization, but the overall response of colonies to experimental manipulation of nutrition remains unstudied in most species (Barrows et al., 1975; Bell, 1974; Breed & Gamboa, 1977). Previous study of the influence of social environment on intranidal behaviour in *C. calcarata* found that colony aggression is reduced in the absence of mothers and DEDs (Huisken et al., 2021). How manipulation of nutrition influences overall behaviour within nests during social cohabitation has not been characterized. Given the direct relationship between nutrition and the production of DEDs, changes to larval nutrition likely alter social behaviour within *C. calcarata* nests.

Here we test the effect of nutritional manipulation on intranidal social behaviour in *C. calcarata* by experimentally enlarging provisions in nests. We predicted that we would observe fewer aggressive behaviours and greater avoidance in control nests possessing DEDs. For the first time within nests of this species, we examine the role of nutritional manipulation in the production of mutual tolerance and the effects of suppressing the production of DEDs on colony cooperative behaviour.

METHODS

Experimental Methods

Observation nests for both control and experimental treatments were constructed using *C. calcarata* nests established in the wild in cut raspberry branches, *Rubus idaeus,* mounted to bamboo stakes with zip-ties. Nests were distributed in Strafford County, New Hampshire, U.S.A. (43°08′N, 70°55′W) and nest occupancy was determined from entrance holes excavated by foundresses in the
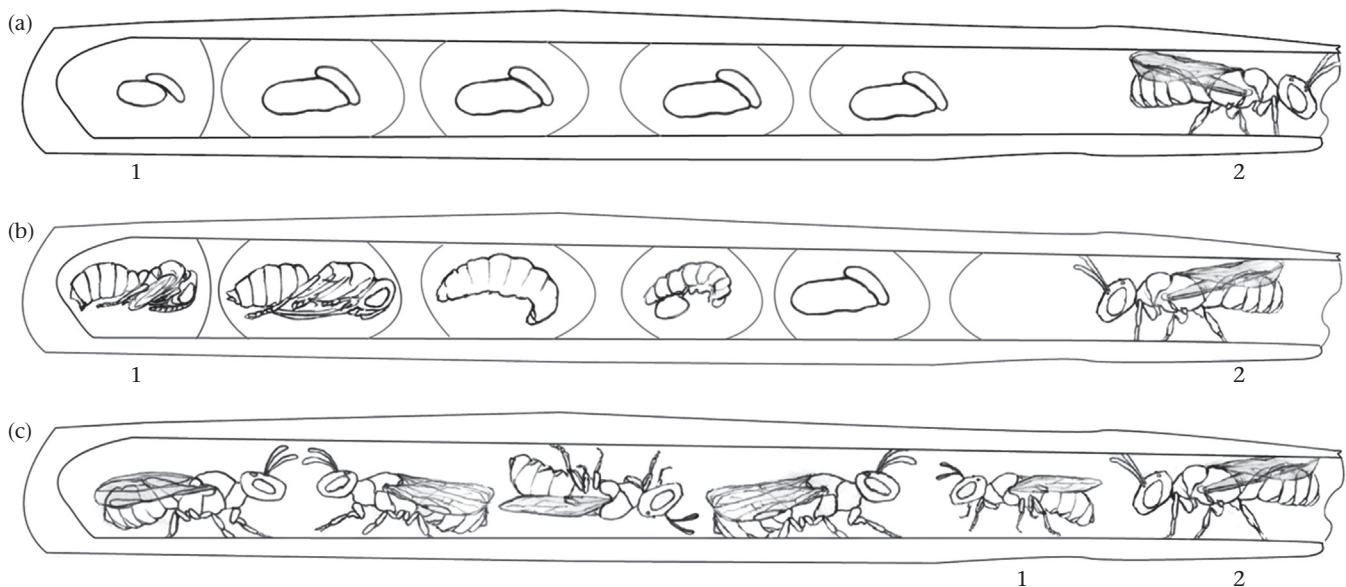

Figure 1. Spring and summer life cycle of *C. calcarata*: (a) spring nests collected in the wild after pollen provisioning and egg laying; (b) mothers continue to associate with nests and (c) in late summer, mothers and dwarf eldest daughters (DEDs) along with regular daughters inhabit nests. 1 = worker-like daughter; 2 = mother. Illustration by Jesse Huisken.

broken ends of stems. Nests were randomly assigned to treatment or control groups. In all nests, brood and pollen were weighed and replaced in the nest. Within treatment nests, the first brood cell, at the terminal end of the linear nest, was subsequently manipulated by increasing pollen provisions with pollen from the last brood cell, closest to the opening of the nest. On average ($\pm$SD), pollen provisions were increased by $26 \pm 8\%$ in mass, approximating the 20% difference in pollen mass received between daughters in a first brood cell and other daughters found in wild nests (Lawson et al., 2016). All stems were then mounted vertically within indoor wooden boxes to reduce ambient light exposure to the nest interior. Transparent plastic cups with domed tops covered each nest opening to create an enclosed foraging arena containing feeding stations (Fig. 2). Food and water were readily available in the foraging cups with fresh flowers and water replaced daily. Nests were longitudinally opened, and a plastic window was applied to exposed nests to facilitate infrared video recording of nest interiors. Brood were allowed to develop normally within nests prior to observation.

Nest interiors were monitored with a Sony HDR SR-11 Hybrid Nightshot, with 50 min observation periods each day between 5 July and 1 September 2018, during the period when brood have begun to reach maturity. Behaviours were scored from previously developed ethograms of this species, including observations of intranidal behaviours, with observers blinded to treatment (Huisken et al., 2021; Withee & Rehan, 2016). These behaviours were then categorized into previously established types, either aggressive or avoidant (Huisken et al., 2021; Rehan & Richards, 2013; Withee & Rehan, 2016). We classed as aggressive three behaviours ('biting', 'nudging' and 'c-posturing') performed when bees present their sting. We classed two behaviours as avoidant ('reversing', when one bee turns and moves away from another bee, and 'backing away' from another bee; Arsenault et al., 2018; Rehan & Richards, 2013). Each behaviour was scored as an event such that frequency and durations of behaviours were also recorded. A total of 26 nests (13 control, 13 nutritionally manipulated treatment nests) were used in this study. A total of 729 of the 50 min observation periods were recorded over 34 days: totalling 366 recordings in control and 363 in treatment conditions (total observation time = 607.5 h). After all behavioural assays were completed, bees were euthanized by freezing at $-20\ ^\circ$C.

Animal Welfare Note

Bees were kept in the laboratory from 5 July to 28 August 2018. Nests were provided with natural light to forage and dark enclosures in their wild established nests. Bees were provided with ample fresh flowers and sugar water and food was changed daily. Bees were not disturbed in the nest, but rather videorecorded using infrared cameras. The only manipulation to nests was done to foraging females that were captured in foraging cups.
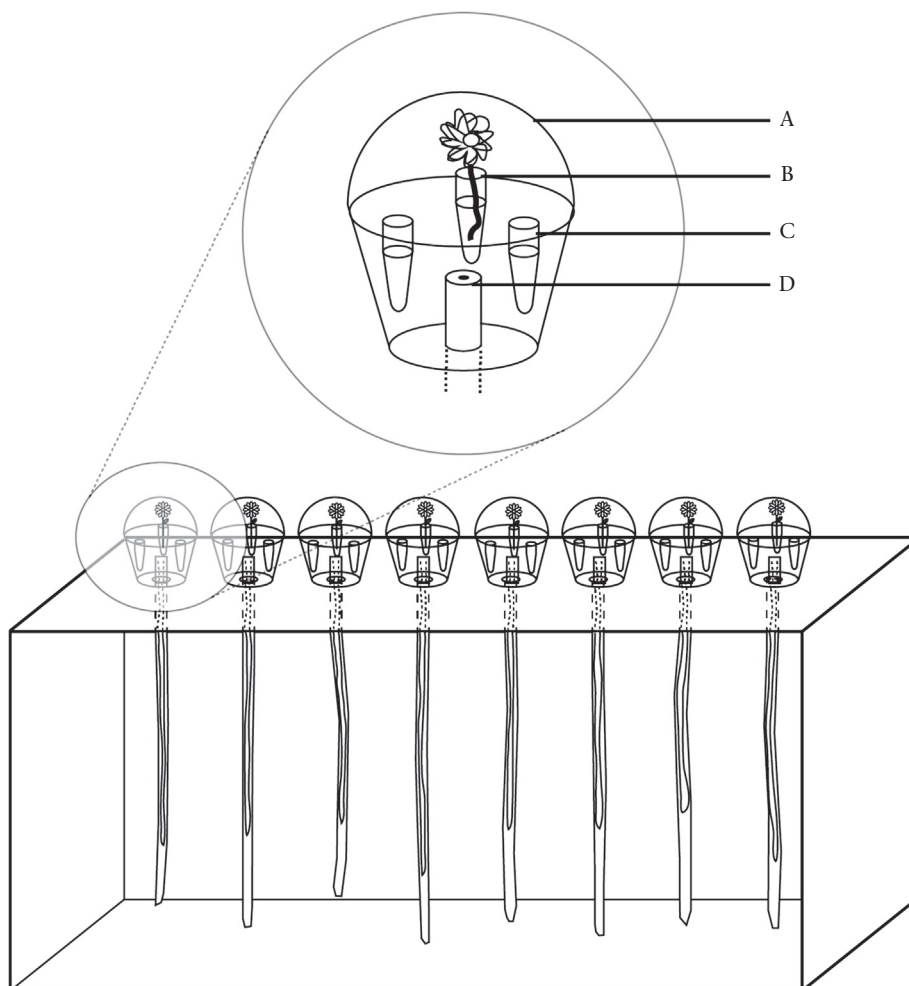

Figure 2. Open observation nestbox. Inset: close-up view of foraging arena, with plastic cup (A), flowers (B), feeding stations with water and sugar water (C), and opening of stem nest (D). A single nestbox contained eight separate nests kept in total darkness, each with a foraging arena exposed to natural light.

After behavioural assay, all bees were euthanized in the most humane way possible, by freezing at −20 °C, and then transferred to 100% ethanol for storage for future genomic work. All work was conducted in accordance with regulations and guidelines established by the Canadian Council for Animal Care and the York University Animal Care Committee. No licenses or permits were required for this research.

Statistical Methods

All statistical tests were conducted in R v.4.0.3. We analysed difference in weights of pollen provisions in treatment and control nests in separate Welch's two-sample *t* tests for the first brood cell and the remaining cells. To model the effect of nutritional manipulation on aggressive and avoidant behaviours in *C. calcarata,* we used generalized linear mixed models (GLMMs) to model the response of each behaviour and behavioural type in the R package 'glmmTMB' (Brooks et al., 2017). Frequencies and durations of behaviours were modelled as a response variable, using Poisson or negative binomial models when overdispersed, with treatment as a fixed effect, nest as random effect and the natural logarithm of number of bees in an observation as offset. Zero-inflation parameters were included where necessary to validate model fit, and diagnostics and visual inspection of residuals were performed in 'DHARMa' to validate all models (Hartig, 2020). Significance of treatment was tested using Wald type II chi-square tests, and a Holm−Bonferroni correction was applied as multiple behaviours were considered from the same data.

RESULTS

First brood cell pollen provisions in control nests were significantly smaller than those in the nutritionally supplemented treatment (mean $\pm$ SE = 16 ± 1 mg versus 20.2 ± 1 mg; $t_{22} = -3.07$, $P = 0.006$). Pollen in remaining brood cells did not differ significantly between treatment and control ($t_{165} = -1.28$, $P = 0.202$). Avoidant behaviours were significantly more frequent under nutritional manipulation ($\chi^2_1 = 5.33, N = 729, P = 0.021$; Fig. 3), with an estimate $\pm$ SE of 0.47 ± 0.13 (confidence interval, CI: 0.22−0.72) avoidant behaviours per bee per 50 min observation compared to 0.20 ± 0.06 (CI: 0.08−0.31) in control nests (Fig. 4). Duration of avoidant behaviours also differed significantly between control and treatment nests ($\chi^2_1 = 8.6, N = 729, P = 0.0034$), with an estimate $\pm$ SE of 8.07 ± 2.24 s (CI: 3.67−12.5) of avoidant behaviours per bee per
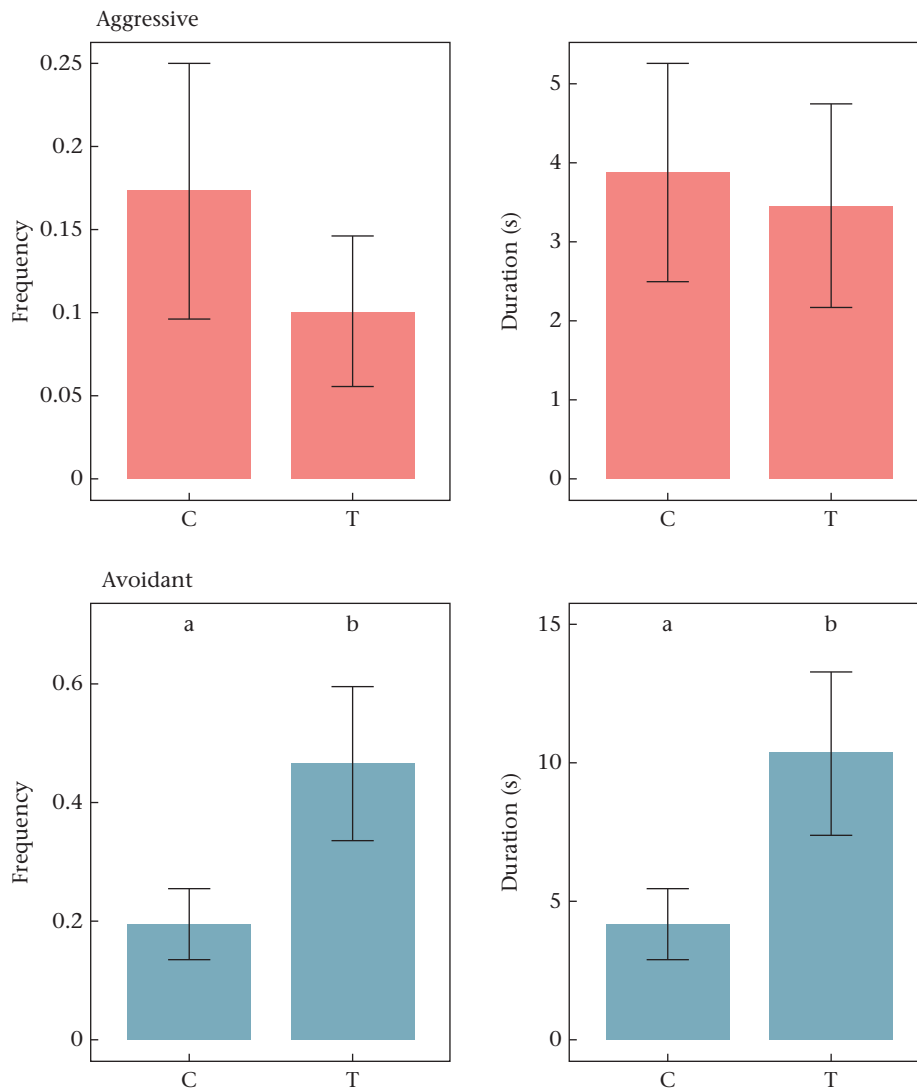

Figure 3. Estimated marginal means ($\pm$ SE) of frequencies and durations of aggressive (top) and avoidant (bottom) behaviours, per bee per 50 min observation, for categories of behaviour by bees in control (C) and treatment (T) nests. Lower case letters indicate statistically significant differences.
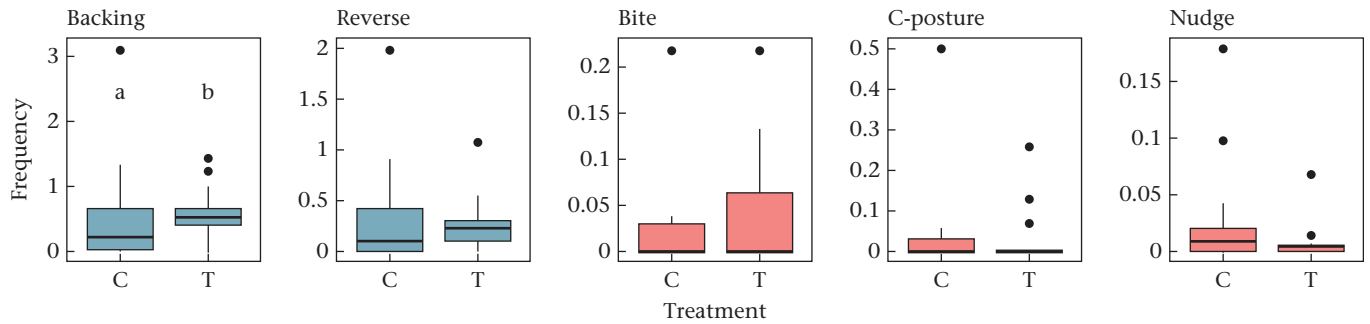
Figure 4. Box plots of frequencies of interactive behaviours per nest ($N = 36$). Differing letters represent significantly different results from GLMM Poisson or negative binomial. C = control; T = treatment; blue = avoidant behaviour; red = aggressive behaviour. Box plots show 25% and 75% quartiles (boxes), medians (lines in the boxes), outermost values within the range of 1.5 times the respective quartiles (whiskers) and outliers (circles).

50 min observation compared to 0.383 ± 0.91 (CI: 1.13–4.69) in control nests. Aggressive behaviours did not significantly differ in overall frequency between treatments ($\chi^2_1 = 0.76$, $N = 729$, $P = 0.384$) or in duration ($\chi^2_1 = 0.077$, $N = 729$, $P = 0.782$).

When frequencies of each behaviour were considered (Table 1), 'backing' was significantly greater in treatments than in control nests (Fig. 4). Durations of both avoidant behaviours, backing and reversing, were also significantly greater in treatment nests (Table 1, Fig. 3).

DISCUSSION

Our experimental manipulation of *C. calcarata* nests demonstrates that alteration of nutrition has an influence on behaviour within nests. While levels of aggression were similar between control and treatment, we found a greater frequency and duration of avoidance in nutritionally supplemented colonies. This confirms previous findings that behaviour in *C. calcarata* and other Hymenoptera is in part conditioned by nutritional manipulation of size (Hogendoorn & Velthuis, 1999; Rehan & Richards, 2013; Richards & Packer, 1994; Smith et al., 2009). Greater avoidance under the nutritional supplementation is largely explained by backing behaviour, whereby bees move rapidly backwards after frontally encountering another bee (Withee & Rehan, 2016). While the observed increase in avoidant behaviour in our study could have been the result of larger bees negotiating the confines of the nest, this is unlikely as treatment nests possessed only a single additional larger individual, and regular daughters frequently pass one another in nests without difficulty (Huisken et al., 2021).

Within some species of eusocial Hymenoptera, backing occurs when a subordinate is led to a location in the nest by a dominant individual (Breed & Gamboa, 1977), but within other species, particularly those lacking complex nests, backing behaviour is exclusively an avoidant behaviour (Rehan & Richards, 2013; Smith et al., 2009). Previous manipulations of social environment in *C. calcarata* by removing mothers, or mothers and DEDs, indicate that DEDs alone do not substitute for mothers in maintaining social structure within nests (Huisken et al., 2021). As DEDs are more

subordinate than regular daughters and more likely to receive aggressive behaviours (Lawson et al., 2017; Rehan & Richards, 2013), the greater expression of avoidance behaviour when first brood cells are nutritionally supplemented is thus likely the result of a loss of social hierarchy among size-matched daughters (Crespi & Ragsdale, 2000; Withee & Rehan, 2016). This would tend to confirm previous findings that regular daughters become more avoidant and will forage on their own behalf in the absence of mothers and worker-like daughters (Huisken et al., 2021).

The experimental nutritional supplement and the effective absence of DEDs led to an increase in avoidant behaviour within the social environment of the nest. This points to the need for further study of how and whether cooperation among siblings emerges in the context of age- and size-based hierarchies. It remains to be determined how division of labour may be decided among regular siblings in the absence of significant size differences, including the role of nutrition during adult stages, through trophallaxis and foraging. Further study of intranidal social behaviours considering individual bees using identifying marks distinguishable under infrared light is also necessary. Although a larger body size improves a bee's chances of dominance in initial aggressive encounters with unfamiliar bees, it is unknown how body size influences dominance in aggressive encounters during longer periods of cohabitation with familiar, related bees (Withee & Rehan, 2016).

Advanced eusocial Hymenoptera exhibit diverse processes, leading to individuals adopting different social roles, including behavioural interactions and more derived traits, such as pheromonal signals (reviewed in Robinson, 1992). Honey bees transition from nursing to foraging specialization throughout their life spans, mediated by behavioural and pheromonal interactions with colony brood and the queen (reviewed in Robinson, 1992). In colonies of primitively eusocial Hymenoptera, all individuals remain totipotent but adopt different tasks within the colony, including egg laying, guarding and foraging (Michener, 1974). Size, larval provisioning and dominance hierarchies, rather than task specialization, may all play a role in reproductive versus worker status in primitively eusocial sweat bees (Kukuk & May, 1991; Richards & Packer, 1994). However, in primitively eusocial wasps, tasks such as foraging and guarding may be determined behaviourally, through social hierarchies arising from repeated aggressive interactions, as well as through age (Bridge & Field, 2007; Pratte, 1989; Unnikrishnan & Gadagkar, 2017; West-Eberhard, 1969). Given that social experience influences the outcome of aggressive contests in *C. calcarata*, it is likely that age and experience interact to determine individual roles in this species (Withee & Rehan, 2016, 2017).

Subordinate social behaviours associated with sibling helping are also found in highly diverse vertebrate taxa, including some species of cooperatively breeding birds and several mammal and fish species (Alexander et al., 1991; Emlen, 1984; Naef & Taborsky,

Table 1
Results of Wald type II chi-square tests GLMMs of frequencies of behaviours per individual bee, and of durations of behaviours

Category	Behaviour	Frequency		Duration	
		χ^2_1	P	χ^2_1	P
Aggressive	Bite	2.05	0.152	1.31	0.253
	C-posture	0.002	0.964	0.002	0.966
	Nudge	1.36	0.243	0.302	0.583
Avoidant	Backing	5.00	0.025	6.48	0.011
	Reverse	2.98	0.084	3.07	0.022

2020; Reyer et al., 1986; Stacey & Koenig, 1990). Within these species, helping is typically flexible, as it is associated with changes in physical development, maturation and reproduction (Creel et al., 1992; Solomon & French, 1997; Taborsky, 1985). For instance, helpers in cichlid fish may be rejected depending on their size and the stage of the breeding pairs' reproductive cycle (Taborsky, 1985). In contrast, for *C. calcarata* and other mass-provisioning Hymenoptera, adult size is directly associated with larval provisions and reproduction is limited to a single annual life cycle, making nutrition a potentially decisive factor in determining social roles and overall social behaviour within a colony (Boomsma & Eickwort, 1993; Lawson et al., 2016, 2017; Packer & Knerer, 1985; Quezada-Euán et al., 2011).

This present study is a significant contribution to understanding how mutual tolerance responds to nutritional manipulation, and thus how size-based social hierarchies may have evolved from ancestral solitary living. Within simple social systems, changes in nutrition alter overall social dynamics in colonies, which can produce more mutually tolerant or avoidant behaviours. Further research is needed to understand how division of labour is determined in a species lacking morphological castes. In particular, the interacting roles of order or eclosion, size-based response thresholds, age and social experience on adult feeding and task allocation are important directions for future studies.

Author Contributions

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