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SHORT COMMUNICATION

Behavioural effects of juvenile hormone and their influence on division of labour in leaf-cutting ant societies

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ABSTRACT

Division of labour in social insects represents a major evolutionary transition, but the physiological mechanisms that regulate this are still little understood. Experimental work with honey bees, and correlational analyses in other social insects, have implicated juvenile hormone (JH) as a regulatory factor, but direct experimental evidence of behavioural effects of JH in social insects is generally lacking. Here, we used experimental manipulation of JH to show that raised JH levels in leaf-cutting ants results in workers becoming more active, phototactic and threat responsive, and engaging in more extranidal activity – behavioural changes that we show are all characteristic of the transition from intranidal work to foraging. These behavioural effects on division of labour suggest that the JH mediation of behaviour occurs across multiple independent evolutions of eusociality, and may be a key endocrine regulator of the division of labour which has produced the remarkable ecological and evolutionary success of social insects.

KEY WORDS: Animal behaviour, Endocrine, Behavioural development, Social insect

INTRODUCTION

Division of labour is characteristic of all social groups and is key to the advantages of sociality (Krause and Ruxton, 2002). Division of labour can be transient in some species with simple sociality, or can involve behavioural or morphological specialisation for tasks in other species with more complex sociality. The social insects provide classic examples of such division of labour, with division of labour between reproductive individuals and workers being one of their defining characteristics, and with workers also showing division of labour amongst themselves (Oster and Wilson, 1979). The division of labour within social insect colonies is key to their ecological success and results in them being considered a major transition in evolution (Smith and Szathmary, 1997). In some taxa, primarily some ants and termites, division of labour amongst workers is based on morphological castes, while in all social insects it involves behavioural specialisation, often based on age, but often also including division of labour within age classes (Hölldobler and Wilson, 1990). Understanding the proximate mechanisms that produce the behavioural and morphological division of labour seen in insect societies is therefore one of the key questions in social insect biology, and evolutionary biology in general.

The environmental and physiological mechanisms involved in division of labour have been extensively studied in the honey bee

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model organism, in which larvae fed an amino acid-rich diet of royal jelly are stimulated to continue on a queen developmental pathway while larvae receiving a diet lacking in royal jelly instead switch to a worker developmental pathway (Corona et al., 2007; Kamakura, 2011). The physiological effect of diet on morphological caste determination in honey bees is mediated by juvenile hormone (JH) levels, with JH also mediating the age polyethism and behavioural task propensity of adult workers (Amsalem and Malka, 2014; Schulz et al., 2002; Sullivan et al., 2000). JH has wide-ranging effects in insects (Flatt et al., 2005; Hartfelder, 2000; Nijhout and Wheeler, 1982; Sullivan et al., 2000), and a number of correlational studies have found JH levels to be higher in foragers compared with in-nest workers for several ant, bee and wasp species (Dolezal et al., 2012; Giray et al., 2005; Lengyel et al., 2007; Penick et al., 2011; Shorter and Tibbetts, 2009), suggesting that JH may have a general regulatory function in the division of labour in insect societies. However, it is unclear what behavioural changes produce this age-related switch, and direct experimental evidence for the influence of JH outside of the honey bee model system is lacking. Here, we investigated experimentally the behavioural effects of JH in leaf-cutting ants (which have evolved eusociality independently of bees), by manipulating JH levels. We examined how an increase in JH affects the activity, phototaxis and threat responsiveness of workers, and whether it induces workers to engage in precocious extranidal activity, using similar assays to those which have been used in honey bee studies (e.g. Erber et al., 2006). We then examined whether the behavioural effects of raised JH levels match those for the key switch in division of labour from intranidal to extranidal work.

MATERIALS AND METHODS

The experiment used five colonies of *Acromyrmex octospinosus* (Reich 1793) leaf-cutting ants, a species with large, complex societies of polymorphic workers, in which medium-sized workers care for the brood and mutualistic fungal crop when young, and switch to extranidal foraging and waste management when old (Camargo et al., 2007; Waddington and Hughes, 2010). Colonies were collected in Gamboa, Panama, and kept at 80±5% relative humidity, 26±2°C, and a 12 h:12 h light:dark cycle on a diet of privet leaves (*Ligustrum* spp.), with water provided *ad libitum*. Brood-tending workers of medium size (1.71±0.019 mm head width) and young age from within the fungus gardens were collected and treated topically on the pronotum with either 1 µl of the JH III analogue methoprene (3.3 µg methoprene in 1 µl acetone – ca. 0.223 µg mg^{-1} insect mass) or 1 µl acetone control, three times a week for 2 weeks. During the application period, ants were housed in individual pots. This methoprene dose was similar to, or lower than, that used previously to produce naturally realistic changes in JH for other social insects (ca. 0.06–0.625 µg mg^{-1} insect mass per application; Cahan et al., 2011; Shorter and Tibbetts, 2009; Tibbetts et al., 2013). Two hours after the last application, ants were tested using the behavioural assays detailed below, with different ants being used for each assay. In order to confirm that ants from the two treatment groups were of similar age, a subset of workers (N=41 for control, N=37 for JH) were photographed dorsally and the cuticular darkness (which correlates with age, reliably distinguishing

young workers from old; Armitage and Boomsma, 2010) of the middle third of the rear femur quantified using ImageJ software.

Behavioural assays

To test whether methoprene made ants more active, ants were placed individually in a 90 mm Petri dish lined with filter paper, allowed to acclimatise for 5 min, and then filmed for 10 min using a Logitech c920 webcam (methoprene treatment $N=37$, control $N=35$; 5–6 ants per colony per treatment). Speed of movement was quantified using AntTrak path analysis software (Tranter et al., 2014).

To test whether methoprene affected phototaxis, individual ants were placed in a 90 mm Petri dish, with one half blackened out with tape. After 5 min acclimatisation, ants were filmed for 10 min and the proportion of time spent in the light half of the Petri dish recorded ($N=40$ for both methoprene and control treatments; 8 ants from each colony per treatment).

To test whether methoprene increased the threat responsiveness of ants, we used a mandible opening response (MOR) assay (Norman et al., 2014). Ants were harnessed and their response to a freshly freeze-killed nestmate or non-nestmate (*Acromyrmex echinatior*) worker was tested in a random order. The stimulus was placed in contact with the focal ant's antennae for 10 s, and its responses were recorded as either mandible gaping for a period of >1 s (thereby displaying a threat response) or showing no such response during the 10 s (methoprene $N=37$, control $N=35$; 7–8 ants per colony per treatment).

To test whether methoprene increased extranidal behaviour in a more natural environment, individuals were observed in a mini-nest set-up, consisting of a small piece of fungus in a 60 mm Petri dish, 15 attendant small, young workers to aid fungus care and one larva from the natal colony. The Petri dish was covered to keep the nest environment humid

and dark, and placed in a larger box (240×190×75 mm). One focal ant from each treatment was placed in each mini-nest and left overnight to acclimatise. The locations of each focal ant were then recorded at 30 min intervals for 8 h (methoprene $N=38$, control $N=34$; 6–8 ants per colony per treatment). All assays were then subsequently repeated on 'natural' foragers and nest workers that were observed either within the nest tending to the fungus or outside of the nest collecting leaves, respectively ($N=30$ for both castes, with 6 ants taken from each of the same five colonies of *A. octospinosus*).

Statistical analyses

All data were analysed in IBM SPSS 20.0 (Chicago, IL, USA) using generalized linear mixed models (GLMM) with treatment (methoprene or control) as a fixed factor and colony of origin as a random factor. Model fit was determined using AIC values, with non-significant interaction terms removed to obtain minimum adequate models. The activity (speed of movement), phototaxis (proportion of time spent in the light half of the Petri dish) and behaviour in mini-nests (proportion of time spent engaged in extranidal activity) were analysed with gamma distributions and log link functions, and threat responsiveness of ants (positive MOR or no response) with a binomial distribution and log link function. *Post hoc* pairwise comparisons of treatments were made using the sequential Bonferroni method to control for multiple comparisons.

RESULTS AND DISCUSSION

Ants treated with methoprene were significantly more active than control ants ($F_{1,70}=8.77$; $P=0.004$; Fig. 1A), and spent significantly more time in the lighter half of the Petri dish instead of the darkened half ($F_{1,78}=5.87$; $P=0.018$; Fig. 1B). Methoprene-treated ants were
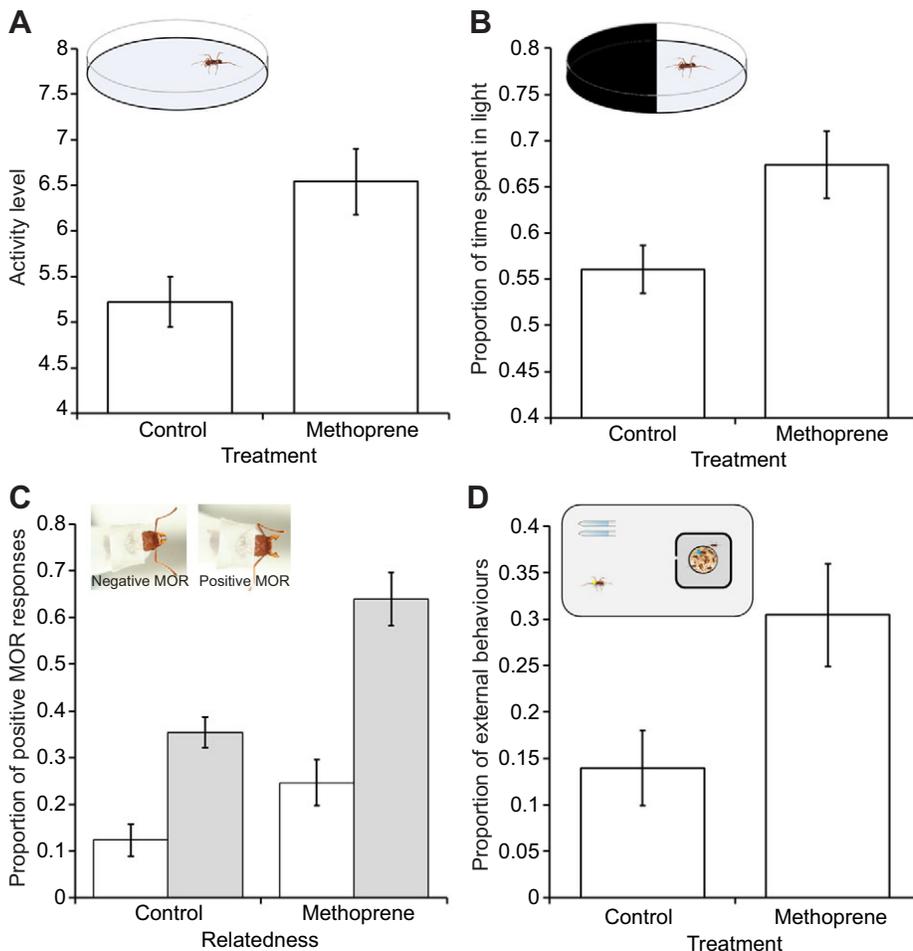
Fig. 1. The effect of increased juvenile hormone (JH) level on ant behaviour. The mean±s.e.m. of behavioural variables measured for young leaf-cutting ant workers from five *Acromyrmex octospinosus* colonies that had been treated for 2 weeks with either the JH analogue methoprene or solvent control. Ants treated with methoprene (A) were significantly more active than control ants (GLMM: $F_{1,70}=8.77$, $P=0.004$, $N=37$ for methoprene, $N=35$ for control), (B) were more phototactic and spent significantly more time in the lighter half of the Petri dish instead of the darkened half (GLMM: $F_{1,78}=5.87$, $P=0.018$, $N=40$ for both methoprene and control), (C) were significantly more threat responsive to both a nestmate (white bars) and a non-nestmate (grey bars) than control ants (GLMM: $F_{1,249}=12.7$, $P<0.001$, $N=37$ for methoprene treatment, $N=35$ for control) and (D) spent significantly more time exhibiting extranidal activity than control ants (GLMM: $F_{1,70}=5.22$, $P=0.025$, $N=38$ for methoprene, $N=34$ for control), illustrated by the yellow marked ant. Intranidal activity is represented by the blue marked ant within the fungus chamber. MOR, mandible opening response.
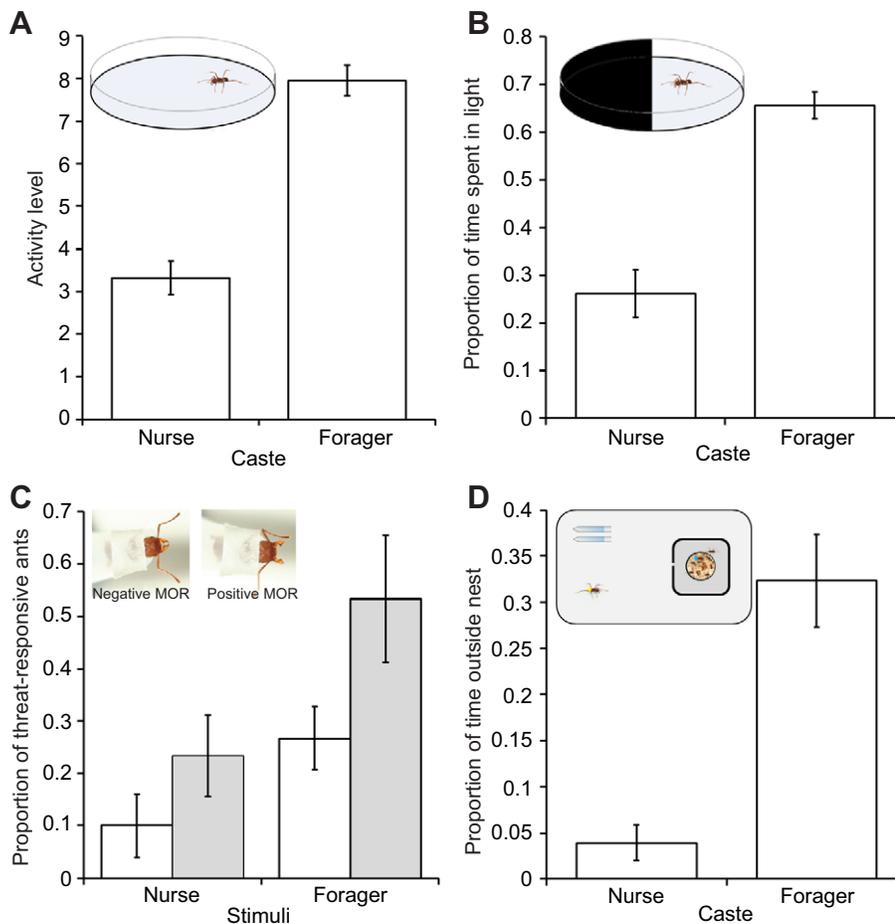
Fig. 2. The behavioural profiles of leaf-cutting ant foragers and nest workers, for comparison with the effects of JH manipulation. The mean± s.e.m. activity for leaf-cutting ant workers that were either foragers (N=30) or in-nest workers (N=30) from five *A. octospinosus* colonies. Foragers were significantly more (A) active (GLMM: $F_{1,58}$=21.2, P<0.001), (B) phototactic (spent significantly more time in the light half of the Petri dish) (GLMM: $F_{1,58}$=48.9, P<0.001) and (C) threat responsive to both a nestmate (white bars) and a non-nestmate (grey bars) (GLMM: $F_{1,117}$=9.94, P=0.002), and (D) showed significantly more extranidal activity compared with in-nest workers (GLMM: $F_{1,1018}$=100.8, P<0.001) (illustrated by the yellow marked ant, compared with a within-nest worker illustrated with a blue mark).

also significantly more threat responsive than control ants, exhibiting almost twice as many positive threat responses to both nestmate and non-nestmate stimuli as the control ants ($F_{1,249}$=12.7, P<0.001; Fig. 1C). Ants from both treatment groups were also more responsive to a stimulus of a non-nestmate compared with a nestmate ($F_{1,249}$=26.3, P<0.001). In the mini-nest experiment, young workers treated with methoprene spent on average twice as much time outside the nest environment as control ants ($F_{1,70}$=5.22; P=0.025; Fig. 1D). No abnormal behaviours were observed from either control or methoprene-treated individuals. Treatment and control ants did not differ in their cuticular coloration and therefore age ($F_{1,76}$=0.275, P=0.601). The effects of methoprene mirror the change seen in leaf-cutting ants when they transition from brood-tending workers to foragers (Fig. 2). Foraging workers were significantly more active ($F_{1,58}$=21.2, P<0.001), more phototactic ($F_{1,58}$=48.9, P<0.001) and more threat responsive ($F_{1,117}$=9.94, P=0.002), and spent significantly more time outside of the nest in a mini-nest set-up ($F_{1,1018}$=100.8, P<0.001) compared with within-nest nurse workers.

The results show that JH level can strongly affect the behavioural profiles of ant workers, and induce them to engage in precocious extranidal activity. Treatment with the JH analogue methoprene caused a significant increase in activity, phototaxis and threat responsiveness of leaf-cutting ant workers, all of which are behavioural characteristics of foraging in the study species, as well as social insects in general (Chapman et al., 2011; Pamminger et al., 2014). Furthermore, there was a doubling in the amount of time that methoprene-treated workers spent engaging in extranidal activities

in a mini-nest setting compared with control ants. JH therefore affects the switch between intranidal and extranidal work that is a key part of the division of labour in ant societies, and it does this by making ants more active, phototactic and threat responsive. This therefore provides evidence for a 'toolkit' situation involving JH, as ants have evolved eusociality independently of bees, indicating that JH regulation of behaviour either is ancestral in eusocial Hymenoptera or has evolved multiple times.

The results are, to our knowledge, the first direct experimental evidence of the effect of JH on division of labour in ant societies, and of the behavioural mechanisms by which this effect is produced. The effects of JH observed on leaf-cutting ants have remarkable similarities with those seen in the best-studied social insect, the honey bee *Apis mellifera*, in which experimentally elevated JH levels lead to precocious foraging (Sullivan et al., 2000), possibly due to the same behavioural mechanisms as we show here in leaf-cutting ants. The results also fit with a number of correlational studies in which JH levels have been found to be higher in foragers of several different ant species, as well as wasps, bees and termites (Dolezal et al., 2009; Giray et al., 2005; Lengyel et al., 2007; Penick et al., 2011). This suggests that JH may have a highly conserved role as a key endocrine mediator of division of labour within eusocial insect societies that has been key to their ecological and evolutionary success.

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