Title: Conserved role of juvenile hormone in regulating behavioural maturation and division of labour in a highly eusocial wasp

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Abstract

Juvenile hormone (JH) in insects plays a key role in regulating reproduction, but in some social insects has also acquired secondary functions in regulating the age-related division of labour among the workers. Previous work has shown that JH regulates division of labour in honeybees, several primitively eusocial paper wasps, and in leafcutter ants, which comprise three independent origins of sociality. As yet, it remains unclear, however, to what degree JH could have a conserved role in regulating division of labour within these clades. To this end, we here investigate if JH also regulates division of labour in the highly eusocial Vespine wasp *Vespula vulgaris*, where previously JH had only been shown to regulate reproduction and affect queen pheromone signalling. In line with JH having a conserved role in regulating division of labour in social wasps, we show that JH in this species does indeed affect behavioural maturation and division of labour in the worker caste. In particular, we show that topical application of the JH analogue methoprene onto workers accelerates behavioural maturation and hastens the age at which workers start to forage, thereby significantly decreasing the time they spend conducting activities inside the nest. We discuss how this conserved role of JH in regulating division of labour could indicate the same conserved genetic toolkits being used to regulate division of labour across several independent origins of sociality. In addition, we examine the implications of JH having several pleiotropic effects in this species and discuss whether this could constrain the expression of reproductive conflicts in their societies.

Keywords: Temporal polyethism, Division of labour, Juvenile Hormone, Social insects, *Vespula vulgaris*

Highlights

- Juvenile hormone regulates the age-related division of labour in the common wasp *Vespula vulgaris*

- The JH analogue methoprene sped up the transition from intranidal tasks to foraging tasks.

- JH has a conserved role in regulating the age-related division of labour across several independent origins of sociality in insects.

Introduction

The ecological success of social insects can in large part be attributed to their advanced social organization and the ways in which the workers divide their labour (Oster & Wilson, 1978; Hölldobler & Wilson, 1990; Duarte et al., 2011). Most commonly, insect societies display an age-related division of labour – also known as "age

polyethism" – whereby young workers perform activities inside the colony and older workers switch to carrying out more risky foraging and nest defence tasks outside the colony (Robinson, 1992; Bourke & Franks, 1996; Tofilski, 2002; Robinson & Jandt, 2021). This age-associated division of labour and progression from less risky to more risky tasks is optimal from a colony-level perspective (Tofilski, 2002), and allows the workers to specialize in different tasks throughout their lives (Lindauer, 1961; Wilson, 1971; Seeley, 1982; Jeanne, 1986; 1991; Seeley, 1995). The species with the best-studied division of labour is the honeybee *Apis mellifera* (Robinson et al., 1989; Robinson, 1992; Huang & Robinson, 1996). Honeybees exhibit a complex age-related pattern of division of labour, with newly emerged bees beginning their lives as nurses, performing tasks primarily inside the hive, and older workers transitioning to outside activities, such as foraging (Winston, 1987; Seeley, 1995). As yet, studies of the division of labour of highly eusocial wasps are scanter. Relative to bees and ants, temporal polyethism in most wasps is considered less pronounced (Brian & Brian, 1952; Potter, 1964; Spradbery, 1973; Akre et al., 1976; Matsuura & Yamane, 1990), as most workers keep on carrying out a variety of tasks over their entire lives (Hurd et al., 2007). At the same time, workers in species such as the common wasp *Vespula vulgaris* still start by performing activities inside the nest, only to later, progress to nest building and riskier tasks such as foraging and nest guarding (Potter, 1964; Hurd et al., 2007). Neotropical caste-flexible swarm-founding wasps of the tribe Epiponini, such as *Polybia occidentalis*, were also found to employ a system of temporal polyethism that was very akin to that of honeybees, with workers abruptly changing task allocation in response to their age (Jeanne et al., 1988; Jeanne et al., 1992; Seeley, 1995), but with task allocation also being influenced to a certain extent by worker size (O'Donnell & Jeanne, 1995). Similarly, in more complex social systems that also evolved eusociality independently, such is the case of termites, it was demonstrated for some species of both lower and higher termites a clear pattern of temporal polyethism (Crosland & Traniello, 1997; Hinze & Leuthold, 1999; Traniello & Leuthold, 2000; Hinze et al., 2002), although there is contrasting evidence showing no effect of age or size in task allocation (Rosengaus & Traniello, 1993).Juvenile hormone (JH) has been pointed out as one of the key regulators of both reproduction and age-related division of labour in eusocial insects (Robinson, 1992; Fahrbach, 1997; Robinson & Vargo, 1997; Bloch et al., 2000; Hartfelder, 2000; Sullivan et al., 2000; Brent et al., 2005; Elliott & Stay, 2007; Shorter & Tibbetts, 2009; Korb, 2015; Norman & Hughes, 2016; Oliveira et al., 2017; Norman et al., 2019; Oi et al., 2021a; Oi et al., 2021b; Ferreira et al., 2022). In solitary insects, JH has long been known to affect a variety of traits, including reproduction, metamorphosis, social behaviour, diurnal cycles, diapause, stress resistance, and aging (Nijhout, 1998; Hartfelder, 2000; Flatt et al., 2005; Jindra et al., 2013; Yamamoto et al., 2013; Li et al., 2019). Over the last decade, studies have shown the importance of JH in

regulating a similarly diverse set of traits in social insects (Montagna et al., 2015; Norman & Hughes, 2016; Pamminger et al., 2016; Oi et al., 2021a; Oi et al., 2021b). In addition, comparative studies have shown how ancestral functions in regulating reproduction were complemented or modified, resulting in JH also regulating other aspects of their behaviour (Fahrbach, 1997; Robinson & Vargo, 1997; Hartfelder, 2000; Shorter & Tibbetts, 2009; Oi et al., 2021a; Prato et al., 2021). A gonadotropic effect of JH, i.e. a function in regulating reproduction, is based on the hormone stimulating vitellogenin synthesis in the fat body and promoting its uptake in the ovarian follicles (reviewed in Wyatt & Davey, 1996; Raikhel et al., 2005; Santos et al., 2019). In both primitively and advanced eusocial species of wasps, bumblebees, and some ants and termites, this gonadotropic function is retained (e.g. Brent et al., 2005; Elliott & Stay, 2007; Korb et al., 2009; Shpigler et al., 2014; Korb, 2015; Norman et al., 2019; Oi et al., 2021a; reviewed in Oi et al., 2021b; Prato et al., 2021; Ferreira et al., 2022), but in some species, this is complemented by other functions linked to reproductive regulation, including reproductive competition through dominance and aggression (Pandey et al., 2020; reviewed in Oi et al., 2021b) as well as the production of queen pheromones and fertility-linked cues on the eggs or cuticle(Oi et al., 2021a; reviewed in Brent et al., 2016; Oi et al., 2021b; Ferreira et al., 2022) or in determining reproductive fate (Lüscher, 1972; Okot-Kotber, 1985; Cornette et al., 2008; Korb et al., 2009; reviewed in Korb, 2015).

Besides this gonadotropic function, it was shown or suspected that in primitively eusocial wasps and some ant species, JH also regulates division of labour (West-Eberhard, 1996; Giray et al., 2005; Lengyel et al., 2007; Shorter & Tibbetts, 2009; Dolezal et al., 2012; Penick et al., 2012; Norman & Hughes, 2016). In fact, in the advanced eusocial honeybee *A. mellifera*, the original gonadotropic function of JH is lost and only this derived function of JH in regulating division of labour is retained (Robinson, 1985; 1987, Cameron & Robinson, 1990; Fahrbach, 1997; Robinson & Vargo, 1997; reviewed in Hartfelder, 2000; Sullivan et al., 2000; Bloch et al., 2002; Schulz et al., 2002), although JH retained gonadotropic functions mainly during the larval development, especially pronounced in queen larvae (Rachinsky & Hartfelder, 1990; Capella & Hartfelder, 1998; reviewed in Hartfelder, 2000). These examples show that significant uncertainty remains about how JH regulates reproduction and behaviour in social insects, and how some of these functions were remoulded in the transition to advanced eusociality.

In the present study, we aim to test whether the role of JH in regulating division of labour, documented in primitively eusocial and caste-flexible wasps (O'Donnell & Jeanne, 1993; Giray et al., 2005; Shorter & Tibbetts, 2009; Prato et al., 2021), was preserved during the transition to an advanced eusocial lifestyle, characterized by the presence of the morphologically specialized queen and worker castes. To this end, we perform experiments

with the common wasp *Vespula vulgaris*, and treat workers with the JH analogue methoprene or the anti-JH precocene to determine if and how this affects age-related patterns of division of labour. Previously, studies on *Vespula* wasps have shown that JH has a gonadotropic function in both queens and workers (Oliveira et al., 2017; Oi et al., 2021a) and also regulates the production of queen pheromones and queen egg-marking pheromones (Oliveira et al., 2017; Oi et al., 2020; Oi et al., 2021a). Workers treated with the JH analogue methoprene, for example, developed their ovaries and acquired a more queen-like cuticular profile (Oliveira et al., 2017; Oi et al., 2021a). In addition, methoprene-treated workers laid eggs that became more queen-like and succeeded in evading being destroyed or "policed" by nestmate workers (Oi et al., 2015; Oi et al., 2020). Thus, studies in *V. vulgaris* provide evidence for JH pleiotropically controlling several fertility-linked traits, including both reproduction and the production of fertility or caste-related signalling molecules (Oliveira et al., 2017; Oi et al., 2020; Oi et al., 2021a). However, the role of JH in regulating division of labour in highly eusocial species, in the same as it does in other eusocial wasps (O'Donnell & Jeanne, 1993; Giray et al., 2005; Shorter & Tibbetts, 2009; Prato et al., 2021), ants (Norman & Hughes, 2016), honeybees (e.g. Robinson, 1985; 1987; reviewed in Hartfelder, 2000; Sullivan et al., 2000; Bloch et al., 2002; Schulz et al., 2002) remains unknown. In the present study, we carry out hormonal manipulations with the common wasp to test if JH also regulates division of labour, and whether this function has therefore been evolutionarily conserved during the transition to advanced eusociality in vespid wasps. We further discuss the wider implications of such evolutionary conservation, e.g. with respect to supporting the use of conserved genetic toolkits to regulate division of labour across different lineages of social insects (Toth et al., 2010).

Material and Methods

Nest collection and treatments

During July and August 2020, a total of nine nests of *V. vulgaris* were collected in the vicinity of Leuven, Belgium (50° 52′ 46″ N; 4° 42′ 3″ E). The collected colonies were nesting in the ground, in attics, or in garden sheds, and were transported to the research facilities of the Laboratory of Socioecology and Social Evolution (LSSE) where they were immediately anesthetized with carbon dioxide (CO_2). We verified that the mother queen was present in all colonies and counted the number of workers present, to be included as a variable 'original colony size' in later statistical analyses (Table S1). All colonies were also verified to be healthy and without parasites and contained combs with brood at various stages of development. Foragers and workers collected inside the colony, excluding

newly emerged ones (recognizable by their lighter pigmentation), were used as older workers in our experiment. The presence of older workers is essential as it allows the colony to keep on functioning normally. Combs were kept in big boxes along with the queen in a climate room with a 12 hours light cycle at 28˚C to obtain newly emerged individuals that were collected within the first 24 hours after emergence. All the newly emerged workers used for each colony were collected in the same day and divided equally among three treatment groups: a group treated with the JH analogue methoprene, one treated with the anti-JH precocene, and one treated with acetone solvent, to act as a control. Checking for physiological and behavioural effects of methoprene treatment is also a standard assay used in the field to infer wether or not a given trait is under JH control (e.g. O'Donnell & Jeanne, 1993; Giray et al., 2005; Shorter & Tibbetts, 2009; Maekawa et al., 2014; Brent et al., 2016; Norman & Hughes, 2016; Pamminger et al., 2016; Oliveira et al., 2017).Workers of each treatment group along with older workers, were marked with a unique colour mark that was applied on the upper part of the thorax using a non-toxic oil-based acrylic marker (Uni-ball Paint marker PX-21) and placed back in the same host colony from which the brood was derived (number of workers treated per colony and treatment: 20-60, avg. nr. of 42). To immobilize the workers to apply both the colour marking and the treatment solution we used a single small dose of CO_2. The treatment solution consisted of either 5 µl of 20 µg/µl methoprene (SIGMA-ALDRICH, analytical standard) acetone solution, 5 µl of 15 µg/µl precocene (SIGMA-ALDRICH, analytical standard) acetone solution or 5 µl of acetone (VWR chemicals) solvent-only control solution, and was applied with a pipette (Gilson, PIPETMAN® L) onto the abdomen of each worker wasp. The doses of the different treatments were selected based on previous successful studies with *V. vulgaris* (Oi et al. 2020, 2021). Acetone solvent was chosen based both on its use in these successful earlier experiments and its low toxicity, its limited impact on behaviour, physiology or longevity, well established based on studies in other Hymenoptera (O'Donnell & Jeanne, 1993; Giray et al., 2005; Jatsch & Ruther, 2021). A solvent-only blank is also the appropriate control, and its use is standard in the field (Robinson, 1987; Korb et al., 2003; Shorter & Tibbetts, 2009; Penick et al., 2012; Brent et al., 2016; Norman & Hughes, 2016; Oliveira et al., 2017; Norman et al., 2019).

Experimental setup and behavioural observations

Each of our 9 study colonies were placed in experimental boxes consisting of two interconnected wooden boxes with identical measures (28 x 28 x 14 cm), of which one contained the brood combs (one large comb or two small ones placed side by side, containing a similar number of empty cells, eggs, pupae, and larvae) and the mother queen and the other served as a foraging arena (Fig. 1). The box with the brood combs was outfitted with a dark cover and a transparent perspex bottom, to allow observations of division of labour. The foraging box was covered

with a transparent perspex lid, was physically connected with a plastic transparent tube (27 cm long), and contained *ad libitum* water, commercial mealworms, a 50:50 water sugar solution and some paper collected from the nest envelope to serve as a building material. Each experimental colony was provided with an equal number of wasps from the three treatment groups, alongside an equal number of older workers (Table S1) and the mother queen, which was introduced to prevent a breakdown of the colony harmony caused by worker egg-laying (Wenseleers et al., 2004).

For the behavioural observations and to determine whether the JH treatments affect the age-related division of labour, we scored four key behaviours that are commonly performed on the nests of this species (Archer, 1972): cell inspection, larval care, nest building, and foraging. The method used to quantify the behaviour of the individuals in the nest was scan sampling, where the two parts of each colony (comb and foraging arena) were observed from the left to the right for a period of 15 minutes per box, i.e. for a total period of 30 minutes per colony per day, at a random hour between 2 pm and 6 pm, which was their period of maximum activity. All the bahaviours (cell inspection, larval care, nest building, and foraging) observed within the 30 minutes were noted down. These behavioural observations were continued for a total duration of 10 days, which seemed appropriate given that in *V. vulgaris*, workers have a life expectancy of only six to 18 days (Potter, 1964). All movements where the wasps placed their heads and entire bodies into empty cells or cells containing an egg were counted as cell inspection. All the worker activity resulting from visiting cells containing mature larvae and any associated exchange of food between them (trophallaxis) were counted as larval care. All the wasps and movements performed within the foraging arena were counted as foraging activities. Construction of new cells or of protective envelope were both counted as nest construction. At the end of the experiment, on day 11, the wasps were sedated with CO_2 and all the marked wasps were counted, to later infer the mortality during the experiment. Furthermore, all the non-marked wasps were also counted to determine if the number of individuals that emerged during the experimental period could have affected the age-related division of the labour of the treated wasps. To this end, the total number of workers that emerged during the experimental period plus the number of treated and older workers that survived until the end of the experiment were included in the statistical analysis as a covariate 'final colony size' (Table S1). All individuals were freeze-killed at -20 °C.
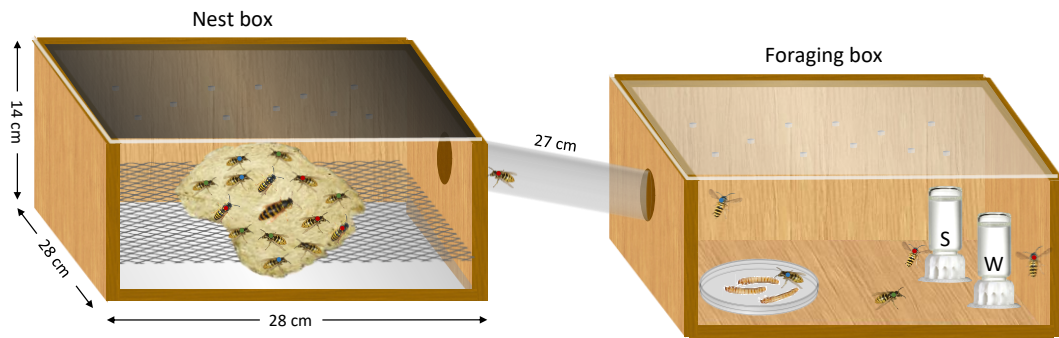
Figure 1. Experimental setup used to study the patterns of age-related division of labour in the highly eusocial Vespine wasp *Vespula vulgaris*. The setup consisted of two boxes interconnected with a plastic tube. One of these served as the nest box and contained one or two brood combs, the mother queen, and an equally sized group of workers treated with various hormone solutions (containing the anti-JH precocene, the JH analogue methoprene or control solvent) and original older workers. The second box served as a foraging arena and contained *ad libitum* sugar water, water, mealworms, and paper collected from the nest envelope so they could continue to construct new cells as well as a protective envelope around the brood combs.

Ethical Note

Vespula vulgaris is a common non-endangered species in Belgium. Nevertheless, nests were delicately collected and quickly transported to the laboratory where they were carefully managed under the controlled use of CO_2. All the conditions for animal welfare were provided and at the end of the experiment wasps were killed by freezing at -20 °C. The field sampling and experiment with live wasps were conducted according to the Belgium and European regulations for animal experimentation (Belgian Royal Decree of 6 April 2010 and European Directive 2010/63/EU on the protection of animals used for scientific purposes of 20 October 2010).

Statistical analysis

To determine how division of labour changed over our experimental period of 10 days across our treatment groups, i.e., methoprene and precocene plus acetone solvent-only treated control workers and untreated old workers, in our 9 study colonies, we fitted multinomial regression models using the *multinom* function of the *nnet* package in R.4.1.1. In this model, the frequency that each task was performed per day (larval care, cell inspection, building, and foraging - based on 30 mins of observation per colony per day) was analysed in function of treatment and day plus their 1st order interaction and with either colony, final colony size, initial colony size or the sum of original colony size and final colony size or the sum of original colony size and initial colony sizeincluded as additional fixed factors. This best-fitting model was selected on the basis of the small Akaike Information Criterion (AICc) value and included day and treatment plus their 1st order interaction effect and colony as fixed factor. Predicted task allocation over time was then calculated as the expected marginal mean proportion of interactions that were of each task group using the *emmeans* package and was displayed using stacked area and line plots. Pairwise post-hoc comparisons of the marginal mean task allocation across treatments were calculated using the same package,

using Tukey adjustment for multiple testing. Finally, the *emtrends* function of the *emmeans* package was used to calculate contrasts in the rate of increase of the different tasks over time across treatments, again using Tukey adjustment for multiple testing.

To check for any differences in mortality across treatments we compared the proportion of treated workers that died over a period of 10 days across our 9 study colonies using a Binomial Generalized Linear Mixed Model (GLMM) fit with *lme4*'s *glmer* function in R 4.1.1. The best model was again selected based on the AICc value and included treatment group and original and colony size as main effects, colony ID as a random intercept, and an observation-level random effect to account for any overdispersion. Pairwise post-hoc comparisons were performed using the *emmeans* package, using Tukey *p*-value adjustment to correct for multiple testing.

Results

The influence of Juvenile Hormone on the age-related division of labour

The multinomial fits to the observed task frequencies show that the rate of progression to engage in particular tasks was significantly different across treatments, thereby demonstrating that JH regulates the age-related division of labour (Figs. 2 & S1, Table 1). In particular, workers treated with the JH analogue methoprene showed a significantly faster progression to foraging (Table 1a) and displayed a higher allocation to foraging behaviour at the end of the experiment at 10 days (Table 1b) compared to the solvent-only control treatment and in the anti-JH precocene treatment (Figs. 2 & S1). The reverse was true for cell inspection, with the methoprene-treated workers showing a faster decrease over time (Table 1a) and displaying a reduced allocation to this task at the end of the experiment (Table 1b) compared to the control and precocene treatments (Figs. 2 & S1). Hence, the JH analogue methoprene significantly accelerated the transition to foraging and decreased the allocation to the intranidal task of cell inspection. We did not find any other significant changes in either nest building or larval care behaviour among the remaining pairwise post-hoc comparisons, including precocene (Table 1). However, this may partly be the result of the fact that nest building behaviour in our experimental setup was only rarely observed. In *V. vulgaris,* it is known that construction of the protective envelope is influenced by temperature, nest site and light intensity (Edwards, 1980) and it is likely that the optimal laboratory conditions we provided in terms of temperature and lighting made it unnecessary for the workers to construct new nest envelope. In the control treatment, it was clear that there was some a progression from larval care to cell inspection, foraging, and nest building, even though temporal polyethism overall was not pronounced (Fig. 2). For the older workers

present in the colony, task allocation remained virtually constant across the experimental period, perhaps because older workers comprised wasps from several cohorts' stages in the colony (Fig. 2). Similar results were obtained when the analysis was performed without the old workers (Table S2).
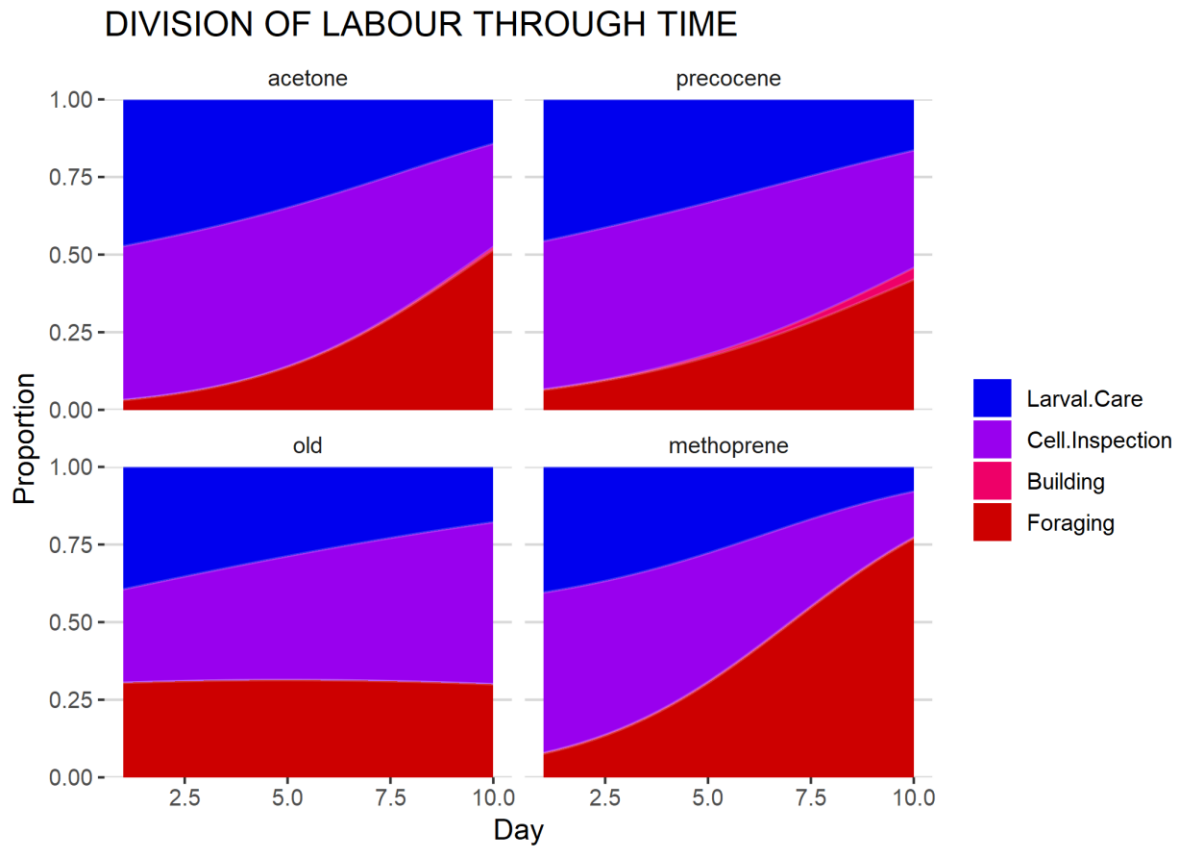
Figure 2. Patterns of division of labour through time across different hormonal treatment conditions in the common wasp *Vespula vulgaris*. Stacked area charts are based on a multinomial model fit to the relative frequency of selected tasks being performed in each treatment condition over the experimental period of 10 days (full model with terms shown in Table 1). Foraging Behaviour and Cell Inspection make up a significantly larger and smaller proportion of the interactions in the methoprene treatment than in the other conditions, acetone, precocene and old workers, and show a significantly steeper increase and decrease, respectively, over time (Table 1).

Table 1. Effect of hormonal treatment on division of labour in the common wasp *Vespula vulgaris*, based on a multinomial model fit to observed task frequency over time across the full duration of our experiment of 10 days. In this best-fitting model, selected on the basis of the AICc value, colony, as well as day and treatment plus their interaction, were included as fixed effects. (A) Contrasts in marginal trends, i.e. in the increase in the log of the odds to engage in each task over time (calculated using the *emtrends* function). (B) Contrasts in the marginal means, i.e. in the log of the odds to engage in each task at the last day of our experiment, at day 10 (calculated using the *emmeans* function). Estimates, Standard Errors (SE), Lower and Upper 95% Confidence Limits (CL) and Tukey-corrected *p-values* are shown. Foraging Behaviour and Cell Inspection showed a significantly steeper increase and decrease, respectively, over time in the methoprene treatment (A) and made up a significantly larger and smaller proportion of the interactions at the end of the experiment (B).

A) Contrast in marginal trends	Task	Estimate	S.E.	Lower 95 % CL	Upper 95% CL	p-value

	Task	Estimate	S.E.	Lower 95 % CL	Upper 95% CL	p-value
Acetone -Methoprene	Larval Care	0.005	0.014	-0.033	0.042	0.742
Acetone - Old	Larval Care	-0.014	0.015	-0.053	0.026	0.358
Ace - Precocene	Larval Care	-0.004	0.013	-0.039	0.030	0.743
Methoprene-Old	Larval Care	-0.018	0.016	-0.061	0.024	0.253
Methoprene-Precocene	Larval Care	-0.009	0.015	-0.048	0.030	0.537
Old - precocene	Larval Care	0.009	0.015	-0.031	0.050	0.540
Acetone -Methoprene	Cell Inspection	0.034	0.016	-0.006	0.075	0.03
Acetone - Old	Cell Inspection	-0.038	0.016	-0.080	0.004	0.02
Ace - Precocene	Cell Inspection	-0.004	0.014	-0.040	0.031	0.753
Methoprene-Old	Cell Inspection	-0.072	0.018	-0.119	-0.025	5.31e-5
Methoprene-Precocene	Cell Inspection	-0.039	0.016	-0.081	0.003	0.02
Old - precocene	Cell Inspection	0.034	0.016	-0.009	0.077	0.04
Acetone -Methoprene	Building	0.001	0.002	-0.004	0.005	0.734
Acetone - Old	Building	0.001	0.001	-0.002	0.004	0.388
Ace - Precocene	Building	-0.002	0.003	-0.009	0.005	0.393
Methoprene-Old	Building	0.000	0.002	-0.004	0.004	0.863
Methoprene-Precocene	Building	-0.003	0.003	-0.011	0.005	0.337
Old - precocene	Building	-0.003	0.003	-0.010	0.004	0.243
Acetone -Methoprene	Foraging	-0.040	0.017	-0.083	0.004	0.02
Acetone - Old	Foraging	0.051	0.014	0.013	0.088	3.84e-04
Ace - Precocene	Foraging	0.011	0.011	-0.017	0.039	0.308
Methoprene-Old	Foraging	0.091	0.020	0.039	0.142	3.61e-06
Methoprene-Precocene	Foraging	0.051	0.017	0.005	0.096	0.003
Old - precocene	Foraging	-0.040	0.015	-0.079	0.000	0.008
B) Contrasts in marginal means at day 10	**Task**	**Estimate**	**S.E.**	**Lower 95 % CL**	**Upper 95% CL**	*p-value*
Acetone -Methoprene	Larval Care	0.064	0.046	-0.055	0.182	0.512
Acetone - Old	Larval Care	-0.036	0.062	-0.194	0.123	0.939
Ace - Precocene	Larval Care	-0.022	0.050	-0.151	0.108	0.973
Methoprene-Old	Larval Care	-0.099	0.062	-0.258	0.060	0.379
Methoprene-Precocene	Larval Care	-0.085	0.052	-0.218	0.047	0.350
Old - precocene	Larval Care	0.014	0.066	-0.155	0.183	0.997
Acetone -Methoprene	Cell Inspection	0.184	0.069	0.007	0.362	0.04
Acetone - Old	Cell Inspection	-0.190	0.090	-0.421	0.041	0.150
Ace - Precocene	Cell Inspection	-0.046	0.076	-0.241	0.150	0.932
Methoprene-Old	Cell Inspection	-0.374	0.088	-0.600	-0.148	1.22e-4
Methoprene-Precocene	Cell Inspection	-0.230	0.075	-0.423	-0.037	0.01
Old - precocene	Cell Inspection	0.144	0.094	-0.097	0.386	0.417

Acetone -Methoprene	Building	0.006	0.017	-0.038	0.050	0.984
Acetone - Old	Building	0.010	0.016	-0.031	0.050	0.925
Ace - Precocene	Building	-0.028	0.042	-0.136	0.079	0.904
Methoprene-Old	Building	0.004	0.008	-0.016	0.024	0.966
Methoprene-Precocene	Building	-0.035	0.042	-0.142	0.073	0.841
Old - precocene	Building	-0.038	0.042	-0.146	0.069	0.797
Acetone -Methoprene	Foraging	-0.254	0.087	-0.477	-0.031	0.02
Acetone - Old	Foraging	0.215	0.093	-0.022	0.453	0.092
Ace - Precocene	Foraging	0.096	0.089	-0.132	0.324	0.703
Methoprene-Old	Foraging	0.470	0.093	0.230	0.709	2.87e-06
Methoprene-Precocene	Foraging	0.350	0.092	0.114	0.586	0.001
Old - precocene	Foraging	-0.120	0.096	-0.367	0.128	0.600

Mortality during the experiment

A binomial GLMM shows that the worker mortality significantly differed across treatments (Anova type III test, Table 2), with worker mortality being significantly higher among the methoprene treated workers and the old workers than among the control workers (Fig. 3, Table 2). In the old workers, this was likely simply due to their old age when compared to the newly emerged treated wasps (Figs. 2, S1), while in the methoprene treated workers toxicity effect could have contributed to the observed mortality. Importantly, however, there were no significant differences in worker mortality between the methoprene and precocene treatments (Fig. 3, Table 2). Hence, mortality differences could not have contributed to the observed difference in division of labour between these treatments shown above. Workers obtained from original colonies with larger sizes exhibited significantly reduced worker mortality (Fig. 4, Table 2).
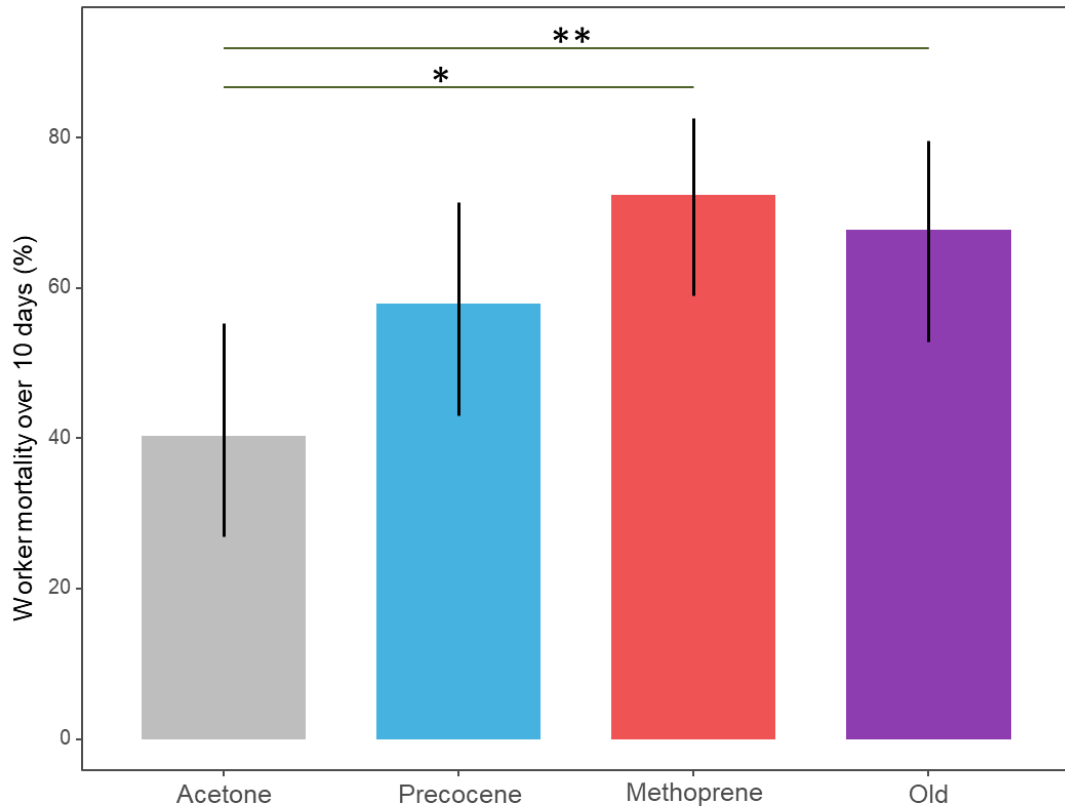
Figure 3. Effect of the different hormonal treatments on the observed worker mortality over the duration of our experiment of 10 days. The effect plot is derived from a binomial GLMM to the proportion of marked workers that died over the duration of the experiment (full model with terms shown in Table 2). Significance codes for Tukey corrected pairwise posthoc tests: $p < 0.001$ ***, $p < 0.01$ **, $p < 0.05$ *.
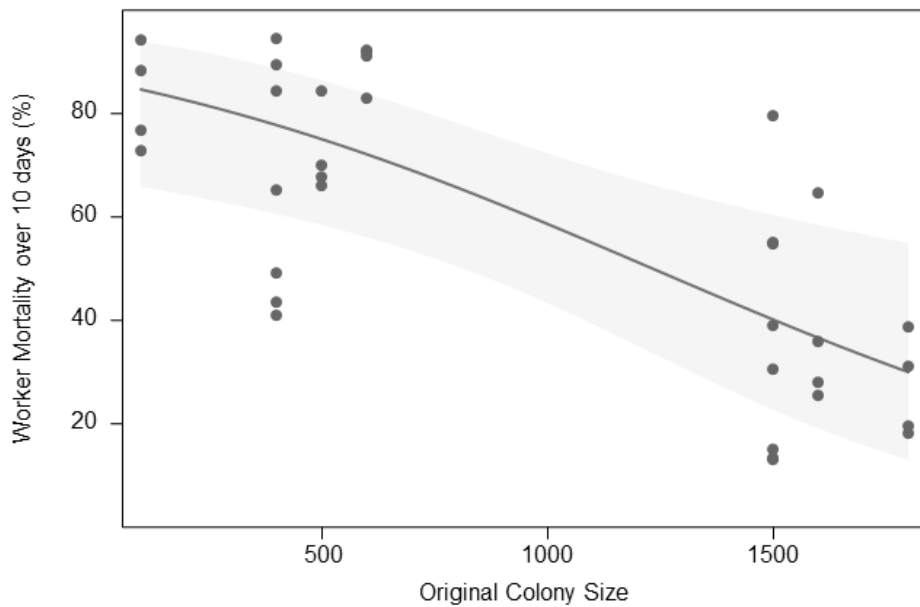
Figure 4. Effects of original colony size (colonies from which the combs were derived) on observed worker mortality over the full duration of the experiment (10 days), based on a binomial GLMM analysis (full model with terms shown in Table 2). Dots represent the partial residuals. Experimental colonies derived from colonies with a larger original size experienced significantly less worker mortality (*p-value* = 0.013*).

Table 2. Results of a Binomial GLMM to test for differential effects of the hormonal treatments on worker mortality over the duration of the experiment (10 days). The Binomial GLMM analyses the proportion of marked workers that died during the experiment in function of treatment, and included nest coded as a random intercept and an observation-level random effect added to take into account overdispersion and original colony size (of the original colony from which the experimental colony was derived) as covariates. (A) Anova Type III tests, (B) Pairwise Tukey posthoc tests (odds ratio for workers to die are shown). Significance codes: *p < 0.001 ***, p < 0.01 **, p < 0.05 *.*

(A) Anova Type III	χ^2	d.f.	p-value				
(Intercept)	1.494	1	0.099				
Treatment	14.331	3	0.002	**			
scale(Colony_size_original)	6.1602	1	0.013	*			
(B) Contrasts	Odds ratio	S.E.	Lower 95 % CL	Upper 95% CL	z ratio	p-value	
Methoprene - Acetone	3.915	1.496	1.467	10.450	3.572	0.002	**
Old - Acetone	3.072	1.227	1.101	8.570	2.810	0.026	*
Old - Methoprene	0.785	0.309	0.285	2.160	-0.616	0.927	
Precocene - Acetone	2.054	0.786	0.768	5.490	1.879	0.237	
Precocene - Methoprene	0.525	0.199	0.198	1.390	-1.704	0.322	
Precocene - Old	0.669	0.264	0.243	1.840	-1.020	0.734	

Discussion

Overall, our results are the first to demonstrate that also in highly eusocial Vespine wasps, JH hastened the onset of foraging behaviour. In particular, we showed that in the highly eusocial common wasp *Vespula vulgaris*, experimental application of the JH analogue methoprene onto workers sped up the transition from intranidal tasks such as cell inspection, to foraging-related tasks and increased the overall proportion of the workers engaged in foraging behaviour compared to in a control solvent-only treated group or a group treated with the anti-JH precocene. We further showed that this result could not be caused by differential mortality and stress induced by the different hormonal treatments, as there were no significant differences in worker mortality across the methoprene and precocene-treated worker groups, and previous studies have successfully applied similar doses of both treatments (Oi et al. 2020, 2021).

Precocene did not display any effect on the behaviour of the treated wasps. This contrasts to some extent with the result that precocene treatment did slightly affect the cuticular chemical profiles associated with age- related tasks in the swarm-founding wasp *Polybia occidentalis* (Prato et al., 2021), even though strictly speaking no direct behavioural effect could be demonstrated there either. In social Hymenoptera, the behavioural effects of precocene have been best documented in bumblebees, where it affects aggressiveness and dominance (Amsalem et al., 2014; Pandey et al., 2020), There is ageneral lack of knowledge regarding the effects of precocene on the behaviour

and neurophysiology in insects, along with the ideal concentrations and number of applications that are necessary to supress, inhibit or even destroy the corpora allata. In *Drosophila*, for example, multiple applications of precocene are required for lasting effects, as the corpora allata can recover after a single precocene application round (Wilson et al., 1983). However, the use of higher precocene doses can also induce increased mortality (Rembold et al., 1979; Fluri, 1983). Furthermore, the reason why precocene has a much more subtle effect than methoprene can be also linked with their different modes of action (Haunerland & Bowers, 1985; Charles et al., 2011; Jindra et al., 2013; Amsalem et al., 2014).

Overall, our results have several major implications. First, they suggest that JH-regulated division of labour is evolutionarily conserved in eusocial Hymenoptera, not only across primitively and advanced eusocial wasps (O'Donnell & Jeanne, 1993; Giray et al., 2005; Shorter & Tibbetts, 2009), but potentially even among several independently evolved social insect lineages with an age-related division of labour, including wasps (cf. supra), honeybees (Robinson, 1985; 1987; reviewed in Hartfelder, 2000; Sullivan et al., 2000; Bloch et al., 2002; Schulz et al., 2002) and ants (Norman & Hughes, 2016). Second, our results confirm that JH has various distinct pleiotropic effects in the common wasp, with JH regulating not only reproduction and fertility-related traits, such as the production of queen and queen egg-marking pheromones, as shown previously (Oi et al., 2015; Oliveira et al., 2017; Oi et al., 2020; Oi et al., 2021b), but also mediating division of labour. In the sections below we will expand in more detail on each of these points.

Shared regulation of division of labour by JH across several social insect lineages

The fact that experimental application of the JH analogue methoprene stimulated workers of the highly eusocial common wasp *V. vulgaris* to switch from carrying out intranidal to extranidal foraging-related tasks mirrors earlier results showing that JH regulates the age-related division of labour in the primitively eusocial Polistini paper wasps *Polistes canadensis* (Giray et al., 2005) and *P. dominulus* (Shorter & Tibbetts, 2009) and in the caste-flexible Epiponini wasp *Polybia occidentalis* (O'Donnell & Jeanne, 1993; Prato et al., 2021). In fact, in wasps, so far only a single study on the primitively eusocial paper wasp *Ropalidia marginata* could not detect any effect of JH on division of labour (Agrahari & Gadagkar, 2003; reviewed in Oi et al., 2021a). Altogether, these results indicate that the role of JH in regulating division of labour may be relatively well conserved across both primitively and highly eusocial wasps. Even more remarkable is that the role of JH in regulating division of labour has also been experimentally established in several other species belonging to different independently evolved lineages of social Hymenoptera that display an age-related division of labour, including the honeybee *A. mellifera*

(e.g. Robinson, 1985; 1987; reviewed in Hartfelder, 2000; Sullivan et al., 2000; Bloch et al., 2002; Schulz et al., 2002) and the leaf-cutting ant *Acromyrmex octospinosus* (Norman & Hughes, 2016), and correlational evidence of JH titres being higher in foragers than nurses also exists for the ants *Pogonomyrmex californicus*, *Myrmicaria eumenoides* and *Harpegnathos saltator* (Lengyel et al., 2007; Dolezal et al., 2012; Penick et al., 2012). This suggests that the underlying gene regulatory networks that control division of labour on which JH acts are evolutionarily conserved, potentially by being derived from conserved genetic pathways that regulate the life cycle of pre-social ancestors. This "genetic toolkit" or "reproductive groundplan" hypothesis has been suggested to explain convergent evolution of a range of social traits built from the same ancestral building blocks across different social lineages (Amdam et al., 2006; Page & Amdam, 2007; Toth & Robinson, 2007; Johnson & Linksvayer, 2010; Jandt & Toth, 2015; Toth & Rehan, 2017). Although JH was shown to have a gonadrotropic role (Brent et al., 2005; Elliott & Stay, 2007), on the cuticular hydrocarbons (Brent et al., 2016) and a strong influence on reproductive caste fate in social Blattodea (Lüscher, 1972; Okot-Kotber, 1985; Cornette et al., 2008; Korb et al., 2009; reviewed by Korb, 2015), recent insigths obtained from the extraction of JH titers seem to discard the possibility of JH being involved in the regulation of age related division of labour in termites (Elsner et al., 2021).This further suggests that the fuction of JH in regulating age related division of labour was possibly acquired later during insect evolution, as migth not be present in the more basal social Blattodea (Elsner et al., 2021).

With respect to division of labour among social insect workers, Toth and Robinson (2007) have suggested that the insulin/insulin growth factor I signalling (IIS)– a key integrative pathway regulating nutrition, fertility and aging in solitary species – has been co-opted as a conserved toolkit from solitary species to regulate division of labour in social species (*Apis mellifera*, Ament et al., 2008, *Polistes metricus*, Daugherty et al., 2011). Furthermore, it is also known that as a direct or indirect response to IIS, the corpora allata in solitary insects produce juvenile hormone (Tatar et al., 2003), thereby also providing a link between insulin signalling and JH.

Variation in the pleiotropic effects of JH across different social insect lineages

An ancestral gonadotropic function of JH is well supported based on work with solitary insects (reviewed in Hartfelder, 2000; Wasielewski et al., 2011; Tibbetts et al., 2013; Kapheim, 2017; Kapheim & Johnson, 2017,), and is conserved across most social wasps (Oliveira et al., 2017; Oi et al., 2021b; Prato et al., 2021 ; reviewed in Oi et al., 2021a) and bumblebees (Röseler & Röseler, 1986; 1988; Shpigler et al., 2014; reviewed in Hartfelder, 2000; Pandey & Bloch, 2015). Derived additional functions of JH in social insects in driving fertility and queen

signalling (Van Oystaeyen et al., 2014; Oliveira et al., 2017; Oi et al., 2021a; Oi et al., 2021b; Prato et al., 2021; Prezoto et al., 2021) and in regulating division of labour (cf. supra) are also relatively well conserved across several social insect lineages that independently evolved sociality. Likewise, JH in some ant species regulates reproduction, division of labour and drives task-related and caste-related cuticular hydrocarbon profiles, even if evidence is more scattered (Wheeler & Nijhout, 1981; Penick et al., 2012; Norman & Hughes, 2016; Pamminger et al., 2016). In contrast honeybees have lost its gonadotropic functions and instead mainly regulates age-related division of labour (Robinson, 1985; 1987; Cameron & Robinson, 1990; Fahrbach, 1997; Robinson & Vargo, 1997; reviewed in Hartfelder, 2000; Sullivan et al., 2000; Bloch et al., 2002; Schulz et al., 2002). The extent to which reproduction and social behaviour are co-regulated by JH can vary, implying that these pleiotropic links are somewhat flexible, and can still be altered in function of lineage-specific selection pressures.Future research should include the measurement of the JH titers as well as the search for JH signalling genes and gene regulatory changes after the use of the manipulations with JH treatments to further explore the effects and mechanisms of action of JH.

Authors' contributions

Conceptualization: C.A.O.; Methodology: H.M.F., V.D.P., C.A.O.; Formal analysis: H.M.F., V.D.P., T.W. Writing- review & editing: C.A.O., H.M.F., V.D.P., T.W.; Visualization: T.W. Supervision: C.A.O.; Project administration: C.A.O., T.W.; Funding Acquisition: C.A.O, T.W.

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