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RESEARCH ARTICLE

Sex- and caste-specific developmental responses to juvenile hormone in an ant with maternal caste determination

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ABSTRACT

Juvenile hormone is considered to be a master regulator of polyphenism in social insects. In the ant *Cardiocondyla obscurior*, whether a female egg develops into a queen or a worker is determined maternally and caste-specific differentiation occurs in embryos, so that queens and workers can be distinguished in a non-invasive manner from late embryogenesis onwards. This ant also exhibits two male morphs – winged and wingless males. Here, we used topical treatment with juvenile hormone III and its synthetic analogue methoprene, a method that influences caste determination and differentiation in some ant species, to investigate whether hormone manipulation affects the development and growth of male, queen- and worker-destined embryos and larvae. We found no effect of hormone treatment on female caste ratios or body sizes in any of the treated stages, even though individuals reacted to heightened hormone availability with increased expression of *krüppel-homolog 1*, a conserved JH first-response gene. In contrast, hormone treatment resulted in the emergence of significantly larger males, although male morph fate was not affected. These results show that in *C. obscurior*, maternal caste determination leads to irreversible and highly canalized caste-specific development and growth.

KEY WORDS: Social insects, Caste determination, Developmental plasticity, Polyphenism, Methoprene

INTRODUCTION

Queen-worker caste polyphenism in social Hymenoptera (ants, bees, wasps) is a prime example of developmental plasticity. In ants, various factors can be associated with caste-specific development, including genotype (Helms-Cahan et al., 2002; Julian et al., 2002; Pearcy et al., 2004), maternal effects (Bier, 1952; Passera, 1980; Schwander et al., 2008), and the abiotic and biotic environment (e.g. Wesson, 1940; Brian, 1954, 1955, 1973; Vargo and Passera, 1992; Penick and Liebig, 2012). To explain how intrinsic and extrinsic factors are translated into differential caste development, Wheeler proposed juvenile hormone (JH) as a master regulator (Wheeler, 1986), inspired by work on other insects showing that JH, together with ecdysone, controls the duration of development and growth via the timing of moults (Nijhout, 2003), thus making it an important factor in regulating size polymorphism (Nijhout and Wheeler, 1982). For ants, this insight was based on two studies of *Pheidole* spp. soldier caste determination (Wheeler and Nijhout, 1981, 1983) and five studies in three species (*Pheidole pallidula*, *Myrmica rubra*, *Solenopsis invicta*) demonstrating that treatment with juvenile hormone or its synthetic analogues could result in increased queen production (Brian, 1974; Vinson and Robeau, 1974; Robeau and Vinson, 1976; Banks et al., 1978; Passera and Suzzoni, 1979).

Since Wheeler's seminal work, to the best of our knowledge, a mere five additional studies have investigated the relationship between hormone signalling and queen/worker development in ants (Schrempf and Heinze, 2006; Helms-Cahan et al., 2011; Penick et al., 2012; Libbrecht et al., 2013; Kuhn et al., 2018), and only one has followed up on previous reports (de Menten et al., 2005), so that empirical evidence for the generality of JH-mediated regulation of caste and caste-specific body size is still surprisingly limited. One reason for this is the difficulty of studying development in ants: many do not mate in the lab or only produce sexuals when colonies are sufficiently large and, unlike honey bees, ants do not rear castes in distinct brood cells, making it challenging to follow individual development and to identify castes during early development (Schultner and Pulliainen, 2020).

The genus *Cardiocondyla* is one of 10 ant genera in which complete worker sterility has evolved, meaning that adult workers lack reproductive organs. We recently showed that in *Cardiocondyla obscurior*, queen- and worker-destined embryos and larvae can be distinguished in a non-invasive manner and with high accuracy using caste-specific crystalline deposits (Schultner et al., 2023). Along with differences in the presence of crystalline deposits, embryos of the two castes are distinct in their miRNA and gene expression (Oettler et al., 2023 preprint). Final (third) instar larvae also exhibit caste-specific gene expression (Schrader et al., 2015; Klein et al., 2016) and wing disc morphology (Oettler et al., 2019), and castes differ in size from the second larval instar onwards (Schultner et al., 2023). Single-queen colonies produce more queen-destined offspring as queens age (Jaimes-Niño et al., 2022) and workers do not engage in differential rearing or culling according to caste (Schultner et al., 2023). Together, this indicates that female caste in this species is determined very early in development, likely via maternal effects. In addition to the two female castes, *C. obscurior* produces two discrete male morphs – large, winged males and small, wingless males (Kinomura and Yamauchi, 1987; Heinze and Hölldobler, 1993; Oettler et al., 2010). Like female castes, the two male morphs differ in wing disc development (Oettler et al., 2019) and gene expression in the final larval instar (Schrader et al., 2015; Klein et al., 2016). However, male morphs cannot be distinguished visually before this stage and when male morph is determined is unknown, although it is presumed to occur later than in females (Schrempf and Heinze, 2006).

Using this system, we tested (1) how a maternal mode of female caste determination, associated with very early morphological and molecular differentiation, affects the action of JH and the degree of

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flexibility in caste-specific development and (2) whether male and female polyphenism are regulated in a similar manner. We did this by subjecting embryos and larvae to (synthetic) juvenile hormone treatment, which has previously been linked to increased queen and winged male production in *C. obscurior* (Schrempf and Heinze, 2006). We documented effects on adult caste and morph ratios and on larva and adult body sizes, as well as on the expression of the conserved JH-response gene *krüppel-homolog 1*.

MATERIALS AND METHODS

Study species

Cardiocondyla obscurior Wheeler 1929 is a myrmicine tramp ant found in the tropics and subtropics. These small ants live in colonies with a few dozen workers and several queens (Heinze, 2017; Oettler, 2020). Queens, workers and males develop via an egg stage and three larval instars (first instar: L1; second instar: L2, third instar: L3), which can be identified by body shape and melanization of the mandibles (Schrempf and Heinze, 2006). Queen and worker-destined eggs and larvae can be distinguished using caste-specific crystalline deposit patterns (Schultner et al., 2023). All individuals used in this study derived from laboratory stock colonies originally collected in Japan in 2010 and in Brazil in 2009 (Schrader et al., 2014; Errbii et al., 2021; Ün et al., 2021b) and were treated according to 3R principles (https://www.nc3rs.org.uk/). Stock and experimental colonies were kept in 9.6×9.6×3 cm plastic boxes with plastered bottoms. In each colony, a plastic insert nest with three chambers and a microscope slide covered with black foil as a lid was placed on a notch in the plaster to prevent the insert nest from slipping and consequently damaging the ants and brood. To keep workers from escaping, the upper third of the nest was paraffined weekly. Three metal plates were placed inside each colony holding sponges for water supply, honey and insect prey. All colonies were kept in a climate chamber under a 12 h:12 h and 22° C:26°C light:dark cycle at 75% relative humidity and fed three times a week with chopped cockroaches (once per week) or fruit flies (twice per week), honey and water.

Hormone treatment of larvae of unknown caste and sex

In order to replicate the experimental set-up of a previous study (Schrempf and Heinze, 2006), we treated first, second and third instar larvae of unknown sex and caste with 2 µl of either methoprene (Sigma-Aldrich; 1 mg ml^{-1} diluted in 70% ethanol) (L1: n=157, L2: n=95, L3: n=40), juvenile hormone III (JH III) (Sigma-Aldrich; 1 mg ml^{-1} diluted in 70% ethanol) (L1: n=129, L2: n=36, L3: n=24), 70% ethanol (L1: n=224, L2: n=102, L3: n=21), or acetone (L1: n=66, L2: n=107, L3: n=23) using a Hamilton syringe. We also included a handling control (L1: n=468, L2: n=223, L3: n=30). Individuals were collected from several stock colonies and pooled prior to treatment to remove any colony-level effects. Individuals were treated in groups of five and then transferred on filter paper to rearing nests containing workers from stock colonies. We chose to treat brood in groups because the small size of *C. obscurior* makes individual treatment of eggs and larvae difficult. For practical reasons, each group received the same amount (2 µg) of JH III or methoprene, which represents doses of ∼20,000 µg g^{-1} for L1 larvae, 10,000 µg g^{-1} for L2 larvae and 1000 µg g^{-1} for L3 larvae; these doses are far higher than the effective doses used by Wheeler and Nijhout (1981) to induce soldier development in Pheidole (50–250 µg g^{-1} body weight), but about four times lower than those used by Schrempf and Heinze (2006). Rearing colonies contained between 10 and 30 workers (∼1:2 worker:brood ratio) and worker numbers were standardized weekly until no more brood remained. The sex and caste/morph of each emerging individual was documented and survival and caste/morph ratios were calculated. Caste/morph ratios were compared between treatments using Fisher's exact tests in R v.4.2.2 (https://www.r-project.org/).

Hormone treatment of queen- and worker-destined late-stage embryos and larvae

To examine whether juvenile hormone can influence caste-specific growth and development after caste has been determined, queen- and worker-destined eggs and L1, L2, L3 larvae were treated with either methoprene, JH III, ethanol (solvent control) or left untreated (handling control). For better identification of caste-specific crystalline patterns in eggs, these were selected in PBT solution (0.3%). For each developmental stage and caste, individuals were collected from several stock colonies, pooled and then randomly divided into groups of five on filter paper to remove any colony-level effects. Each group of five individuals was topically treated with either 1 µl methoprene (1 mg ml^{-1} diluted in 70% ethanol), 1 µl JH III (1 mg ml^{-1} diluted in 70% ethanol), 1 µl of 70% ethanol, or none of the above, using a Hamilton syringe. We reduced doses compared with the previous experiment to minimize mortality and because we found no effect even with higher doses (see next paragraph). This process was conducted twice per treatment day, resulting in 10 individuals being treated per day, treatment, developmental stage and caste. Following treatment, the 10 individuals were transferred on filter paper to a nest containing 15 adult workers collected from several stock colonies the previous day. Two hours after treatment, two individuals were randomly selected from each experimental colony, individually flash-frozen in liquid nitrogen and stored in −80°C for gene expression analyses (see below). The remaining individuals remained in the experimental nests to be reared to adulthood. A total of 2240 individuals were treated, of which a subset were sampled for gene expression analyses and the rest were allowed to develop until adulthood (n=56 per treatment, developmental stage and caste).

To assess the effect of hormone dose, in a separate experiment we treated L2 queen- and worker-destined larvae with 2 µl of either methoprene (1 mg ml^{-1} diluted in 70% ethanol) (queen-destined: n=10, worker-destined: n=33), JH III (1 mg ml^{-1} diluted in 70% ethanol) (queen-destined: n=15, worker-destined: n=15), 70% ethanol (queen-destined: n=11, worker-destined: n=15) or acetone (queen-destined: n=15, worker-destined: n=20) using a Hamilton syringe. Individuals were collected from several stock colonies, pooled prior to treatment to remove any colony-level effects and treated in groups of five larvae.

Caste and morph ratios

Experimental colonies were observed once a week to record brood survival and developmental stage. Additionally, the number of workers was counted and any dead or missing workers were replaced from stock colonies. Once the first brood of a colony completed pupation, colonies were checked every other day and when melanization set in, colonies were observed daily to ensure that newly emerged, light-coloured adult workers were isolated before they darkened, so as to remain distinguishable from rearing workers. All freshly emerged adults from one experimental colony were transferred together to a smaller round nest with a plastered bottom containing sponges for water supply, a metal plate with honey, and a folded black foil as a shelter, where they were kept for 5–7 days to allow cuticle hardening. The caste and sex of each fully melanized adult was documented and all adults were frozen in

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Eppendorf tubes at −20°C for morphometric measurements. From these data, we calculated survival and caste prediction accuracy for each treatment, developmental stage and caste, and compared these using Fisher's exact tests in R (Table 1). Because worker- and male-destined eggs and larvae exhibit similar crystalline deposit patterns (E.S., pers. obs.), in each treatment a small proportion of individuals developed into males (31 males in total, Table 1). These were excluded prior to survival and caste prediction accuracy analyses. Note that the survival and caste prediction accuracy results from the handling control have been published as supplemental data in a previous study (Schultner et al., 2023); we nevertheless include them here for comparison.

Development time

Development time was defined as the time in days between treatment and emergence of the first adult from each experimental colony. Development time was calculated separately for each treatment, developmental stage and caste. In cases where more than one caste emerged from an experimental colony, development time was calculated for the first hatched individual of each caste. In late-stage embryos, L1 and L2 larvae, development time was compared between castes and treatments using a linear regression with caste and treatment as explanatory variables, as well as an interaction term to account for caste-specific responses to treatment: development time∼caste×treatment. Model fit was assessed using residual tests

Table 1. Survival and caste ratios of queen- and worker-destined embryos and larvae in the ant *Cardiocondyla obscurior* after hormone treatment

Developmental stage/treatment	Crystalline deposit pattern	*n*	Emerged queens	Emerged workers	Emerged males	Survival (females only)	Survival differences between castes		Caste prediction accuracy		
							Odds ratio	*P*	Correct caste predictions	Odds ratio	*P*
Egg											
Handling control	Queen-like	56	9	10	0	33.9%	1.436	0.489	47.4% (9/19)	Infinite	**0.001**
	Worker-like	56	0	17	1	30.9%			100% (17/17)		
Solvent control	Queen-like	56	12	6	1	32.7%	0.506	0.135	66.7% (12/18)	17.837	**0.006**
	Worker-like	56	1	10	0	19.6%			90.9% (10/11)		
JH III	Queen-like	56	8	9	1	30.9%	0.588	0.275	47.1% (8/17)	Infinite	**0.009**
	Worker-like	56	0	11	3	20.8%			100% (11/11)		
Methoprene	Queen-like	56	10	8	0	32.1%	0.774	0.679	55.6% (10/18)	4.747	0.072
	Worker-like	56	3	12	0	26.8%			80% (12/15)		
L1											
Handling control	Queen-like	56	14	1	0	26.8%	1.953	0.112	93.3% (14/15)	Infinite	**<0.001**
	Worker-like	56	0	23	1	41.8%			100% (23/23)		
Solvent control	Queen-like	56	21	1	0	39.3%	0.910	0.846	95.5% (21/22)	139.815	**<0.001**
	Worker-like	56	2	18	2	37.0%			90% (18/20)		
JH III	Queen-like	56	18	3	0	37.5%	0.686	0.422	85.7% (18/21)	Infinite	**<0.001**
	Worker-like	56	0	16	1	29.1%			100% (16/16)		
Methoprene	Queen-like	56	14	0	1	25.5%	1.583	0.302	100% (14/14)	Infinite	**<0.001**
	Worker-like	56	1	18	2	35.2%			94.7% (18/19)		
L2											
Handling control	Queen-like	56	25	1	0	46.4%	1.284	0.571	96.2% (25/26)	449.740	**<0.001**
	Worker-like	56	1	28	1	52.7%			96.6% (28/29)		
Solvent control	Queen-like	56	25	0	0	44.6%	1.238	0.703	100% (25/25)	Infinite	**<0.001**
	Worker-like	56	0	27	2	50.0%			100% (27/27)		
JH III	Queen-like	56	27	1	0	50.0%	0.965	1.000	96.4% (27/28)	433.828	**<0.001**
	Worker-like	56	1	26	1	49.1%			96.3% (26/27)		
Methoprene	Queen-like	56	24	5	0	51.8%	0.778	0.571	82.8% (24/29)	99.444	**<0.001**
	Worker-like	56	1	24	1	45.5%			96% (24/25)		
L3											
Handling control	Queen-like	56	43	1	0	78.6%	0.191	**<0.001**	97.7% (43/44)	Infinite	**<0.001**
	Worker-like	56	0	22	2	40.7%			100% (22/22)		
Solvent control	Queen-like	56	38	3	0	73.2%	0.260	**<0.001**	92.7% (38/41)	Infinite	**<0.001**
	Worker-like	56	0	21	5	41.2%			100% (21/21)		
JH III	Queen-like	56	27	1	0	50.0%	1.037	1.000	96.4% (27/28)	Infinite	**<0.001**
	Worker-like	56	0	28	1	50.9%			100% (28/28)		
Methoprene	Queen-like	56	31	1	0	57.1%	0.487	0.082	96.9% (31/32)	Infinite	**<0.001**
	Worker-like	56	0	20	5	39.2%			100% (20/20)		
All stages combined											
Handling control	Queen-like	224	91	13	0	46.4%	0.821	0.339	87.5% (91/104)	588.536	**<0.001**
	Worker-like	224	1	90	5	41.6%			98.9% (90/91)		
Solvent control	Queen-like	224	96	10	1	47.5%	0.642	**0.026**	90.6% (96/106)	226.455	**<0.001**
	Worker-like	224	3	76	9	36.7%			96.2% (76/79)		
JH III	Queen-like	224	80	14	1	42.2%	0.828	0.333	85.1% (80/94)	434.405	**<0.001**
	Worker-like	224	1	81	6	37.6%			98.8% (81/82)		
Methoprene	Queen-like	224	79	14	1	41.7%	0.806	0.283	84.9% (79/93)	79.515	**<0.001**
	Worker-like	224	5	74	8	36.6%			93.7% (74/79)		

JH III, juvenile hormone III; L1, 1st instar larva; L2, 2nd instar larva; L3, 3rd instar larva.

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implemented in the DHarma package in R (https://cran.r-project.org/package=DHARMa). Tukey-corrected pairwise *P*-values were calculated using the emmeans package in R (https://CRAN.R-project.org/package=emmeans). In L3 larvae, Kruskal–Wallis tests (function kruskal.test in R v. 4.2.2) were used to test the effect of caste and treatment on development time separately, as the data did not follow a normal distribution.

Body size measurements

To assess the effect of hormone treatment on adult body size, all adults which emerged in experimental colonies were dried under a stereomicroscope light and pinned for subsequent measurements (Table 1). Pinned individuals were photographed at 200× using a Keyence stereomicroscope connected to a camera (Keyence VHX). Head width (HW), head length (HL), mesosoma width (MW), mesosoma length (ML) and petiole width (PW) were measured from photographs after appropriate scaling using ImageJ. In the cases in which adult female caste did not align with presumed caste based on crystalline deposit patterns [queens: 12.8% (51/397) of individuals; workers: 3% (10/331) of individuals; Table 1], the emerging adults were nonetheless measured and included in subsequent analyses. Similarly, all emerged males were measured and included in analyses. The final data set contained 759 individuals (queens – late-stage embryos: handling control, *n*=9; solvent control, *n*=13; JH III, *n*=8; methoprene, *n*=13; L1 larvae: handling control, *n*=14; solvent control, *n*=23; JH III, *n*=18; methoprene, *n*=15; L2 larvae: handling control, *n*=26; solvent control, *n*=25; JH III, *n*=28; methoprene, *n*=25; L3 larvae: handling control, *n*=43; solvent control, *n*=38; JH III, *n*=27; methoprene, *n*=31; workers – late-stage embryos: handling control, *n*=27; solvent control, *n*=16; JH III, *n*=20; methoprene, *n*=20; L1 larvae: handling control, *n*=24; solvent control, *n*=19; JH III, *n*=19; methoprene, *n*=18; L2 larvae: handling control, *n*=29; solvent control, *n*=27; JH III, *n*=27; methoprene, *n*=29; L3 larvae: handling control, *n*=23; solvent control, *n*=24; JH III, *n*=29; methoprene, *n*=21; ergatoid males – late-stage embryos: handling control, *n*=1; solvent control, *n*=1; JH III, *n*=4; L1 larvae: handling control, *n*=1; solvent control, *n*=2; JH III, *n*=1; methoprene, *n*=3; L2 larvae: handling control, *n*=1; solvent control, *n*=2; JH III, *n*=1; methoprene, *n*=1; L3 larvae: handling control, *n*=2; solvent control, *n*=5; JH III, *n*=1; methoprene: *n*=5). A principal component analysis (PCA) of the five measured traits was done using the NIPALS algorithm implemented in the pcaMethods package in R v4.2.2 (Stacklies et al., 2007) to estimate missing values. Principal component 1 scores were compared between the three castes (queens, workers, males), the four developmental stages (late-stage embryos, L1, L2, L3 larvae) and between treatments using a linear regression, with caste, treatment and developmental stage as explanatory variables and an interaction term to account for caste-specific responses to treatment: PC score∼caste× treatment+developmental stage. Residual tests implemented in the DHarma package in R v4.2.2 (Hartig, 2020) were used to assess model fit. There was no significant effect of developmental stage on adult body size (Table 2), so this term was removed from the model before pairwise *P*-values were calculated and Tukey-corrected for multiple comparisons using the emmeans package in R (Lenth et al., 2020).

Since the treatment was applied in a single topical application in each developmental stage, it is possible that an effect on body size was diluted over time. To assess the short-term effect of hormonal manipulation, we repeated hormone treatments using only L2 larvae, which were measured once they reached the L3 stage. As in the previous experiment, L2 larvae of each caste were chosen based on crystalline deposit patterns and treated in groups of five with 1 µl of either JH III, methoprene or ethanol as a solvent control, or subjected to a handling control. The five larvae were transferred to a

Table 2. Effects of hormone treatment on body size and development time in the ant *Cardiocondyla obscurior*

Developmental stage treated	*n*	Linear regression		d.f.	Sum of squares	F/X^2	*P*
Larva size							
L2	250	PC 1 score∼	Caste	1	168.19	128.917	**<0.001**
			Treatment	3	13.20	3.373	**0.019**
			Caste×Treatment	6	12.01	3.068	**0.029**
Adult queen, worker and male size							
Late-stage embryo, L1, L2, L3	759	PC 1 score∼	Morph	2	1747.37	709.367	**<0.001**
			Treatment	3	27.84	7.534	**<0.001**
			Developmental stage	3	7.35	1.99	0.114
			Morph×Treatment	6	38.40	5.196	**<0.001**
Early-stage male and female embryo	74	PC 1 score∼	Morph	2	108.53	41.582	**<0.001**
			Treatment	1	3.39	2.599	0.112
			Morph×Treatment	2	3.81	1.461	0.239
Early-stage male and female embryo	74	PC 2 score∼	Morph	2	18.20	14.495	**<0.001**
			Treatment	1	2.35	3.736	0.057
			Morph×Treatment	2	5.81	4.624	**0.013**
Development time							
Late-stage embryo	62	Days to pupation∼	Caste	1	69.50	4.517	**0.038**
			Treatment	3	23.88	0.517	0.672
L1	46	Days to pupation∼	Caste	1	35.61	1.437	0.238
			Treatment	3	21.43	0.288	0.834
L2	55	Days to pupation∼	Caste	1	144.24	16.331	**<0.001**
			Treatment	3	62.98	2.377	0.081
L3*	58	Days to pupation∼	Caste	1	–	0.704	0.401
			Treatment	3	–	4.122	0.248

*For each trait, data were analysed using a single linear regression except for development time of 3rd instar larvae, which was compared between castes and treatments using separate Kruskal–Wallis tests as the data did not follow a normal distribution.

Depending on the analysis, explanatory variables used in linear regressions refer to caste (queens, workers), morph (queens, workers, males), treatment [handling control, solvent control, juvenile hormone III (JH III), methoprene or a subset of these] and developmental stage [embryo, 1st, 2nd, 3rd instar larva (L1, L2, L3)].

nest with 12 adult workers originating from several stock colonies which had been set up one day earlier. This procedure was repeated for a total of 35 L2 larvae per treatment and caste. After treatment, nests were checked every day for moulted larvae and all larvae that had reached the L3 stage were anaesthetized under CO_2 for 4 h. Larvae were then placed on their backs and photographed at 200x using a Keyence stereomicroscope connected to a camera (Keyence VHX). The order in which the pictures were taken was randomized to buffer the effect of time as larvae were more likely to die, lose water (change of body volume) or wake up after a long waiting period. Larval head capsule width, body width and body length were measured after appropriate scaling using ImageJ (v. 1.53 t). As some larvae died or were damaged in the pre-imaging process, the final data set consisted of 29–35 individuals per caste and treatment (Queens - handling control: n= 32, solvent control: n=29, JH III: n=35, methoprene: n=32; Workers - handling control: n=32, solvent control: n=32, JH III: n=30, methoprene: n=28). Like in adults, a principal component analysis including all traits was run using the NIPALS algorithm implemented in the pcaMethods package in R v4.2.2 (Stacklies et al., 2007) to estimate missing values. Principal component 1 scores were then compared between castes and between treatments using a linear regression, with caste and treatment as explanatory variables and an interaction term to account for caste-specific responses to treatment: PC 1 score∼caste×treatment. Residual tests implemented in the DHarma package in R v4.2.2 (Hartig, 2020) were used to test model fit. Pairwise P-values were calculated and Tukey-corrected for multiple comparisons using the emmeans package in R v4.2.2 (Lenth et al., 2020).

Hormone treatment of early-stage embryos

To test whether caste/morph determination and differentiation can be manipulated early in development before caste-specific crystalline deposits are visible, we treated female and male eggs which were at most 24 h old. To obtain female eggs, experimental colonies containing three mated queens of unknown age, 10 adult workers and five worker pupae were set up from stock colonies (n=25 colonies). To obtain male eggs, experimental colonies containing three queen pupae prior to mating, 15 adult workers and five worker pupae were set up from stock colonies (n=40 colonies). Ants exhibit haplodiploid sex determination, so unmated queens only produce male-destined eggs. Experimental colonies were monitored once per week until the first eggs were observed. Once experimental colonies began producing sufficient numbers of eggs, eggs were removed daily to ensure that only eggs which were at most 24 h old were included in experiments. Eggs from several experimental colonies were pooled to remove any colony-level effects and then divided into groups of five on filter paper, after which they were topically treated with either 1 µl of methoprene (1 mg ml^{-1} diluted in 70% ethanol) or 1 µl of 70% ethanol using a Hamilton syringe. This was done in parallel for both sexes to avoid bias. Treated eggs were transferred on filter paper to rearing colonies containing 15 workers randomly collected from stock colonies; rearing colonies were always set up one day before egg transfer to allow workers to acclimate. Each rearing colony contained 5-25 eggs, as the number of treated eggs depended on the fecundity of queens, which is much higher in mated compared to unmated queens and varies over time (E.S., pers. obs.). In total, we treated 650 early-stage male embryos (solvent control: n=325, methoprene: n=325) and 570 early-stage female embryos (solvent control: n=285, methoprene: n=285). Rearing colonies were monitored twice per week and fed in the same manner as stock colonies. Freshly emerged adults were transferred to a smaller nest with a plastered bottom and sponges for water supply, a metal plate with honey, and a folded black foil as a shelter, and kept for 3-5 days to allow cuticle hardening. The caste/morph of each fully melanized adult was documented and all adults were frozen in Eppendorf tubes at −20°C for morphometric measurements. From these data, survival and caste/morph ratios were calculated and survival and treatment effects were analysed using Fisher's exact tests in R v4.2.2.

Body size measurements of adults emerging from treatment of early-stage embryos were obtained as described above for later developmental stages. The final data set consisted of 74 individuals (queens - solvent control: n=1, methoprene: n=4; workers - solvent control: n=24, methoprene: n=24; ergatoid males - solvent control: n=10, methoprene: n=11). A principal component analysis using the NIPALS algorithm to estimate missing values implemented in the pcaMethods package in R (Stacklies et al., 2007) was run on all traits. Principal component 1 and 2 scores were compared between castes and treatments using a linear regression, with caste and treatment as explanatory variables, as well as an interaction term to account for caste-specific responses to treatment: PC 1/2 score∼caste×treatment. Residual tests implemented in the DHarma package in R (https://cran.r-project.org/package=DHARMa) were used to test model fit and pairwise P-values were calculated and Tukey-corrected for multiple comparisons using the emmeans package in R (https://CRAN.R-project.org/package=emmeans).

Gene expression analysis

qPCR was used to assess the effect of sex, caste and treatment on the expression of the JH-response gene *Krüppel-homolog 1* (*kr-h1*). *C. obscurior* sequences for *kr-h1* were retrieved by running a BLAST search of *kr-h1* protein sequences obtained for *Drosophila melanogaster* (CG45074, www.flybase.org) and *Apis mellifera* (GB45427, www.hymenopteragenome.org) against the *C. obscurior* 1.4 genome (Schrader et al., 2014). Intron-spanning primers for the extracted *C. obscurior* ortholog (Cobs_15554) were designed in Geneious Prime vR10 (https://www.geneious.com). Specific primers (*kr-h1* forward primer sequence: CTTGGTGTGCAGCCCGGACC; *kr-h1* reverse primer sequence: ACCGGTACGGATCCTCGCCC) were tested on pooled samples of queen, worker, ergatoid male and winged male DNA in a temperature gradient PCR (60°C, 63°C, 66°C) and PCR products subsequently sequenced to confirm amplification of the desired transcript. Primer efficiency was tested in a five-step dilution series and was confirmed to be within the acceptable range.

To establish a timeline of *kr-h1* expression and to investigate sex-specific responses to JH, second instar male and female larvae (of unknown caste) were collected from several stock colonies, pooled by sex and then treated in groups of five with 2 µl of methoprene (1 mg ml^{-1} diluted in 70% ethanol, female larvae only), JH III (1 mg ml^{-1} diluted in 70% ethanol) or 70% ethanol using a Hamilton syringe. We further included a handling control, which consisted of larvae which were removed from colonies and immediately flash-frozen in liquid nitrogen and stored at −80°C. Female larvae were collected from stock colonies containing mated queens whereas male larvae were collected from colonies containing only unmated queens. After treatment, larvae were moved to rearing nests containing workers from stock colonies, which had been set up the day before. One, two, six and 24 h after treatment, treated larvae were removed from rearing colonies, individually flash-frozen in liquid nitrogen and stored in −80°C. We ensured that only larvae which had been accepted by workers, i.e. which had been moved inside the nest after treatment, were

included. The final sample consisted of 177 females (handling control: 0 h, *n*=19; ethanol: 1 h, *n*=14; 2 h, *n*=11; 6 h, *n*=13; 24 h, *n*=10; JH III: 1 h, *n*=12; 2 h, *n*=12; 6 h, *n*=15; 24 h, *n*=15; methoprene: 1 h, *n*=16; 2 h, *n*=9; 6 h, *n*=13; 24 h, *n*=18) and 111 males (handling control: 0 h, *n*=21; ethanol: 1 h, *n*=13; 2 h, *n*=13; 6 h, *n*=8; 24 h, *n*=6; JH III: 1 h, *n*=13; 2 h, *n*=12; 6 h, *n*=6; 24 h, *n*=19).

To assess caste-specific responses to hormone treatment, *kr-h1* expression was measured in queen- and worker-destined late-stage embryos, L1, L2 and L3 larvae. All samples were collected from the experiment described in 'Hormone manipulations of queen- and worker-destined late-stage embryos and larvae', 2 h after treatment. This timepoint was chosen based on the timeline of *kr-h1* expression in female embryos of unknown caste described above (Fig. 3). The final data set consisted of 5–14 individuals per treatment, developmental stage and caste (Queens – late-stage embryos: handling control, *n*=14; solvent control, *n*=9; JH, *n*=12; methoprene, *n*=10; L1 larvae: handling control, *n*=5; solvent control, *n*=8; JH, *n*=10; methoprene, *n*=10; L2 larvae: handling control, *n*=5; solvent control, *n*=8; JH, *n*=8; methoprene, *n*=8; L3 larvae: handling control, *n*=7; solvent control, *n*=9; JH, *n*=9; methoprene, *n*=9; Workers – late-stage embryos: handling control, *n*=12; solvent control, *n*=10; JH, *n*=9; methoprene, *n*=9; L1 larvae: handling control, *n*=6; solvent control, *n*=7; JH, *n*=7; methoprene, *n*=8; L2 larvae: handling control, *n*=5; solvent control, *n*=6; JH, *n*=8; methoprene, *n*=6; L3 larvae: handling control, *n*=6; solvent control, *n*=9; JH, *n*=7; methoprene, *n*=5).

Each sample was homogenized with ceramic beads in a shaker, total RNA was extracted using a modified protocol of the ReliaPrep™ RNA Cell Miniprep System (Promega, USA), and cDNA was synthesized with the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad, USA), following the manufacturer's instructions. cDNA concentration was measured using the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, USA) and standardized to 1.5–3 ng µl^{-1}, depending on developmental stage. Each qPCR reaction was run in 10 µl (5 µl SYBR green, 3 µl purified H_2O, 0.5 µl forward primer, 0.5 ml reverse primer, 1 µl cDNA) in a qPCR machine (CFX, Bio-Rad, USA). Three technical replicates were run for each sample and gene. In addition to *kr-h1* and the housekeeping gene *y45*, in all samples we analysed expression of the two sex-specific *doublesex* isoforms dsx^f and dsx^m, which vary in their expression between males and females (Klein et al., 2016). This was done to identify males in samples collected from stock colonies containing mated queens, as males cannot be distinguished morphologically from workers before the pupal stage. All male samples thus identified were excluded before statistical analyses. For amplification of dsx^F, dsx^M and *y45*, primer sequences designed for a previous study were used (Klein et al., 2016). The housekeeping gene *y45* was used for normalization according to the deltaCq method (Schmittgen and Livak, 2008). For timeline data, differences in *kr-h1* expression between treatments were compared separately for each sex and timepoint with linear regressions, using log-transformed expression as a response and treatment as explanatory variable: *kr-h1* $\text{expression}_{\text{sex,timepoint}} \sim \text{treatment}$. To assess caste-specific responses to treatment, *kr-h1* expression was compared separately for each developmental stage, using log-transformed expression as a response and caste and treatment as explanatory variables: *kr-h1* $\text{expression}_{\text{developmental stage}} \sim \text{caste+treatment}$. For all analyses, model fit was assessed using residual tests implemented in the DHarma package in R and pairwise *P*-values were calculated and Tukey-corrected for multiple comparisons using the R emmeans package.

RESULTS

Hormone treatment does not affect caste or morph ratios in *C. obscurior*

A previous study reported that treatment of *C. obscurior* larvae of unknown sex and caste with the synthetic JH analogue methoprene always resulted in the production of winged morphs – queens and winged males (Schrempf and Heinze, 2006). We first attempted to replicate this result by treating larvae collected from stock colonies without regard to caste-specific crystalline deposits. Compared with a solvent control, methoprene treatment (2 µl of a 1 mg ml^{-1} solution in 70% ethanol) did not have a significant influence on winged morph production in any of the three larval stages (Table S1, Fisher's exact test, ethanol solvent control versus methoprene: L1, *P*=0.241; L2, *P*=0.573; L3, *P*=0.86).

Similarly, experimental hormone treatment had no effect on the caste of emerging females when queen and worker-destined late-stage embryos and larvae were treated separately (Table 1). Across all developmental stages and treatments, presumed queen-destined individuals developed into adult queens in 87% (346/397) of cases and presumed worker-destined individuals developed into workers in 97% (321/331) of cases. In the three larval stages, the accuracy of caste fate prediction based on crystalline deposit patterns was >90% in individuals subjected to a handling control, and this remained similar following treatment with a solvent control as well as after treatment with JH III or methoprene (Table 1). We confirmed the irreversibility of caste determination by treating queen- and worker destined second instar larvae with higher doses of hormone; in all cases, treated individuals developed into the predicted caste (Table S1). Compared with larvae, queen caste was more difficult to predict in eggs, with only around half of untreated, presumed queen-destined eggs developing into queens (Schultner et al., 2023); this proportion did not change after treatment (Table 1). In contrast, all untreated, presumed worker-destined eggs developed into workers, indicating that worker-destined eggs are more easily identified than queen-destined eggs and/or more likely to end up in experiments because they outnumber queen-destined eggs in stock colonies. The accuracy of caste prediction in early developmental stages furthermore appears to be influenced by sampling precision, as a previous study showed higher prediction accuracy rates for queen-destined eggs, but lower rates for queen-destined first instar larvae (Schultner et al., 2023). Caste prediction accuracy was lowest for methoprene-treated worker-destined eggs, but still relatively high at 80% (12/15), suggesting minor, if any, effects of methoprene on caste once it can be identified by caste-specific crystalline deposits. Of the presumed queen- and worker-destined individuals which emerged as adults, 4.1% (31/759) developed into males, the majority in replicates with presumed worker-destined individuals. Male morph fate was not affected by treatment, as only wingless males emerged (Table 1).

Caste ratios were also not affected by treatment of female early-stage embryos (1–24 h old), with 2.9% (1/35) of solvent control-treated eggs developing into queens, compared with 7% (4/57) of methoprene-treated eggs (Fisher's exact test, odds ratio=0.393, *P*=0.646). Methoprene-treated eggs showed slightly higher survival [methoprene, 20% (57/285); solvent control, 12.3% (35/285); Fisher's exact test: odds ratio=1.784, *P*=0.016]. When early-stage male embryos produced by unmated queens were treated, only wingless males emerged in both treatments [solvent control: 100% (10/10), methoprene: 100% (11/11), Fisher's exact test: odds ratio=infinite, *P*=1], and treatment did not affect male survival, which was extremely low [solvent control: 3.1% (10/325), methoprene: 3.4% (11/325), Fisher's exact test: odds ratio=1.103, *P*=1].

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Juvenile hormone analogues such as methoprene can be used to kill insects because high doses of these chemicals perturb development (Troisi and Riddiford, 1974; Tay and Lee, 2014; Nur Aliah et al., 2021). Similarly, in *C. obscurior*, survival of treated brood dropped considerably when high doses (5 mg ml^{-1}) were administered (Schrempf and Heinze, 2006). The moderate dose used here did not have adverse effects on survival in queen- and worker-destined individuals, which was similar between treatments in each of the developmental stages (Table 1). Across all developmental stages and treatments, a higher proportion of queen-destined compared with worker-destined individuals survived until pupation (queen-destined: 44.5% (346/893), worker-destined: 38.1% (321/868); Fisher's exact test, odds ratio=0.770, P=0.008); this was mostly driven by differential survival of castes in the third larval instar (Table 1). Adult workers do not discriminate between developing queens and workers, so differential treatment is unlikely to explain this difference (Schultner et al., 2023). Instead, increased survival of queen-destined larvae may stem from size differences between the two castes (Oettler et al., 2019; Schultner et al., 2023).

Male but not female body size is affected by hormone treatment

Ant body size is strongly associated with caste polyphenism, with queens typically larger than workers [see Trible and Kronauer (2017, 2021a,b) and Abouheif (2021) for a discussion about the role of body size in ant caste development]. Diverse factors have been shown to be associated with body size variation in queens and workers, including genotype, maternal effects, nutrition, as well as social and abiotic environment (e.g. Heinze et al., 2003; Hughes et al., 2003; Bargum et al., 2004; Fjerdingstad, 2005; Schwander et al., 2005; Meunier and Chapuisat, 2009; Linksvayer et al., 2011). How caste-specific queen and worker body sizes are attained is not well understood, but studies focusing on size variation within the worker caste have suggested that methylation of *epidermal growth factor receptor*, which links to JH via the insulin signalling pathway, plays a key role in determining worker size, both in species with distinct worker castes (Alvarado et al., 2015) and in species with monomorphic workers (Renard et al., 2022). JH itself has been implicated in increased body size of *Pheidole* soldiers (Wheeler and Nijhout, 1981, 1983; Rajakumar et al., 2012) and *Pogonomyrmex* and *Camponotus* workers (Helms-Cahan et al., 2011; LeBoeuf et al., 2016).

In *C. obscurior*, hormone manipulations did not result in larger queens or workers. After treatment in the second larval stage, queen-destined third instar larvae were always larger than worker-destined third instar larvae (Fig. 1, Table 2, Table S2), confirming previous results (Schultner et al., 2023). Treatment had no effect on the size of worker larvae. Methoprene-treated queen-destined larvae were smaller than those subjected to a handling control, but there was no difference between any of the other treatments (Fig. 1, Table 2, Table S2).

Adult queens emerging from treatments of late-stage embryos and larvae were also larger than adult workers (Fig. 2, Table 2, Table S3). This was independent of the timing of hormone treatment, i.e. the developmental stage at which individuals were treated, so that data were pooled across developmental stages for subsequent analyses (Table 2). As in larvae, treatment did not result in larger queens or workers (Fig. 2, Table 2, Table S3). Similarly, treatment had no clear effect on development time until pupation in any of the four treated stages (Table 2). Caste affected development time of late-stage embryos and second instar larvae, with queens exhibiting longer development times than workers; there was no

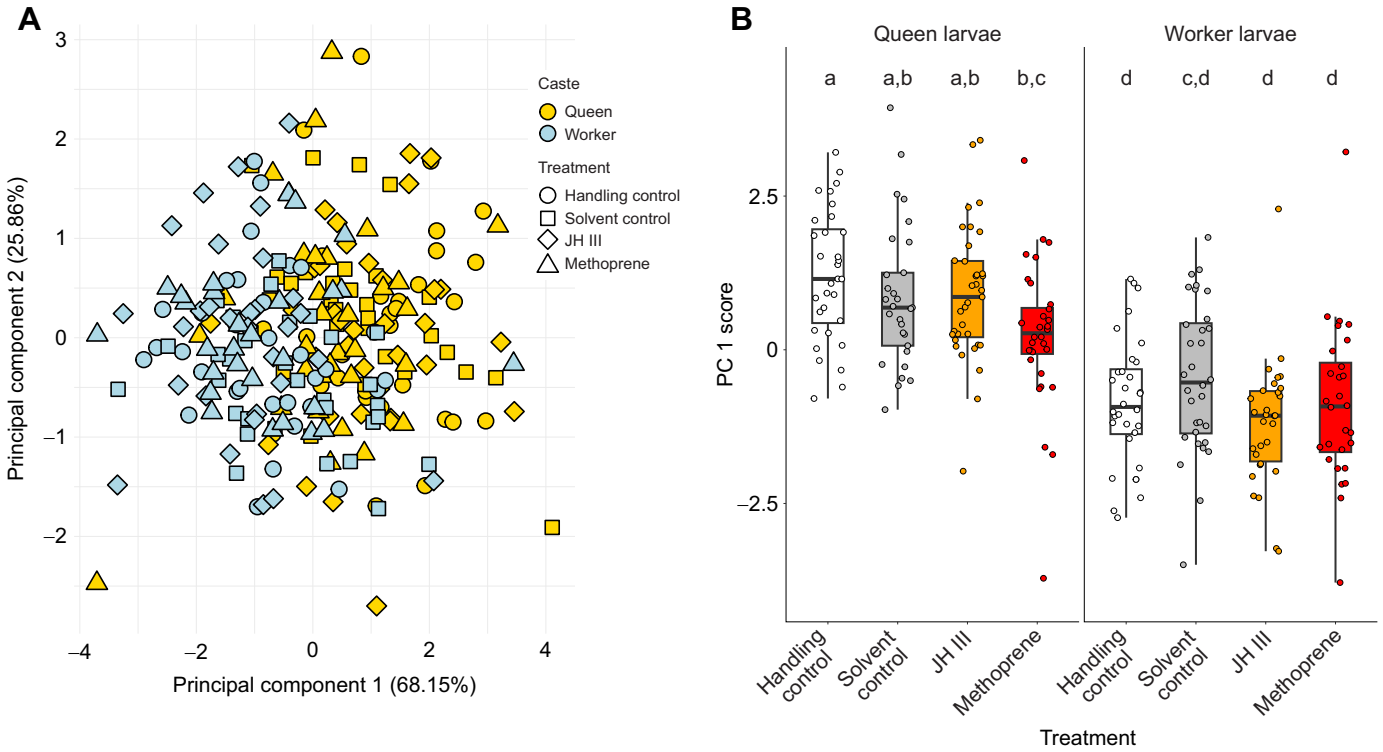

Fig. 1. Body size of queen- and worker-destined larvae in the ant *Cardiocondyla obscurior* after hormone treatment. (A) Principal component analysis of larval head width, body width and body length separates larvae by overall size on PC1. (B) Queen larvae are larger than worker larvae across treatments. Different letters indicate significant differences between groups. See Table S2 for all Tukey-corrected pairwise P-values. JH III, juvenile hormone III.

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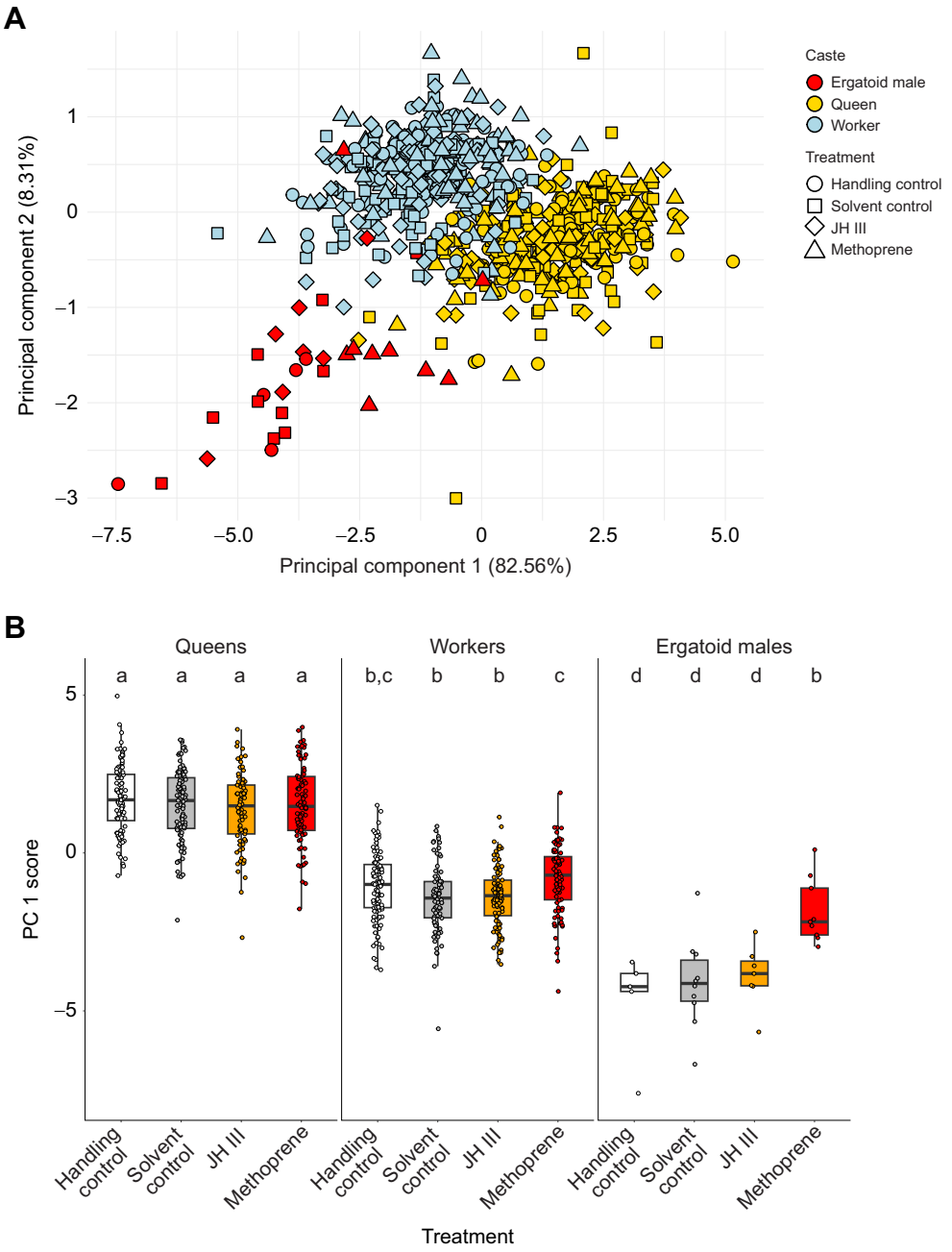

Fig. 2. Body size of adult queens, workers and males emerging from hormone treatment of late-stage embryos and larvae. (A) Principal component analysis separates individuals by size on PC1. (B) PC 1 scores plotted by caste and treatment show that queens are the largest caste, and workers are larger than males. Female size is not affected by hormone treatment, whereas larger males emerge from methoprene treatments. Different letters indicate significant differences between groups. See Table S3 for all Tukey-corrected pairwise *P*-values.

effect of caste on development time of first and third instar larvae (Table 2).

In contrast to females, males treated with methoprene emerged as significantly larger adults than males emerging from the other treatments (Table 2; Fig S1, Table S3). Wingless males are typically smaller than females (Oettler et al., 2019), but males emerging from methoprene treatments exhibited body sizes similar to those of workers (Fig. 2, Table 2; Table S3).

Adult queens emerging from treatment of female early-stage embryos of unknown caste were larger than workers and males, and again, methoprene treatment did not result in larger body size of females (Table 2; Fig S1, Table S4). Male body size did respond to treatment of early-stage embryos, but in an unexpected manner: males emerging from methoprene treatments tended to exhibit decreased thorax lengths and petiole widths compared with solvent-control treated males (Table 2; Fig. S1, Table S4).

Conserved expression of a JH first-response gene in queens, workers and males

Molecular pathways associated with juvenile hormone have been extensively studied in a few model organisms such as the tobacco hornworm *Manduca sexta* and the flour beetle *Tribolium castaneum* (Riddiford, 2012). These have revealed a suite of conserved genes acting downstream of JH, including *methoprene-tolerant* and *krüppel-homolog 1* (*Kr-h1*) (Konopova and Jindra, 2007; Minakuchi et al., 2008, 2009; Jindra et al., 2013). *Kr-h1* expression in social Hymenoptera has mainly been studied in adults, and varies with task in bee workers (Whitfield et al., 2003; Grozinger and Robinson, 2007; Shpigler et al., 2010; Johnson and Jasper, 2016), and with caste (Glastad et al., 2021; Zhang et al., 2021) and reproductive status in ants (Araki et al., 2020; Gospocic et al., 2021). During worker development, pharmacologically induced hypomethylation resulting in increased expression of the growth-promoting *egfr* gene was

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associated with decreased *kr-h1* expression, and an increase in body size (Renard et al., 2022). Similarly, knockout of an oestrogen-related receptor resulted in decreased *kr-h1* expression (Zhang et al., 2021). While these studies have helped confirm the molecular links between insulin and hormone signalling, growth and reproduction, it remains unknown whether the function of *kr-h1* as a JH first-response gene is conserved across sexes and castes in developing ants. We used quantitative PCR to measure *kr-h1* expression following hormone treatment in *C. obscurior*. In female larvae, *kr-h1* expression increased significantly 6 and 24 h after hormone treatment compared with a solvent control (Fig. 3, linear regression, factor: treatment, 1 h: $F_{2,39}$=0.661, P=0.522; 2 h: $F_{2,29}$=2.044, P=0.144; 6 h: $F_{2,38}$=3.997, P=0.027; 24 h: $F_{2,40}$=10.016, P<0.001). In males, hormone treatment led to increased *kr-h1* expression after 1, 2 and 24 h (Fig. 3, linear regression, factor: treatment, 1 h: $F_{1,24}$=6.767, P=0.016; 2 h: $F_{1,23}$=15.993, P<0.001; 6 h: $F_{2,12}$=1.27, P=0.282; 24 h: $F_{1,23}$=4.665, P=0.041).

Krüppel-homolog 1 expression was not affected by the caste of late-stage embryos or larvae (linear regression, factor: caste, eggs: $F_{1,77}$=0.045, P=0.833; L1 larvae: $F_{1,54}$=3.785, P=0.057; L3 larvae: $F_{1,42}$=0.035, P=0.853; L2 larvae: as model residuals did not fit assumptions of normality, linear regressions comparing caste-specific expression were run separately by treatment: handling control: $F_{1,8}$=0.033, P=0.86; solvent control: $F_{1,12}$=0.188, P=0.672; JH III: $F_{1,13}$=4.139, P=0.063; methoprene: $F_{1,10}$=0.032, P=0.862). There was furthermore no effect of treatment in late-stage embryos, first instar and second instar larvae (linear regression, factor: treatment, eggs: $F_{3,77}$=0.522, P=0.668; L1 larvae: $F_{3,54}$=1.934, P=0.135; L2 larvae: as model residuals did not fit assumptions of normality, linear regressions comparing expression by treatment were run separately in each caste: queens: $F_{3,23}$=1.392, P=0.86; workers: $F_{1,20}$=3.016, P=0.054). Third instar larvae of both castes exhibited significantly higher *kr-h1* expression after JH III treatment compared with handling control and solvent control-treated larvae (linear regression, factor: treatment, L3 larvae, $F_{3,42}$=5.574, P=0.002; pairwise-corrected P-values: worker-destined L3 larvae: handling control versus JH III, P=0.037; solvent control versus JH III, P=0.027; queen-destined L3 larvae: handling control versus JH III, P=0.037; solvent control versus JH III, P=0.027).

DISCUSSION

Juvenile hormone studies in ants have employed a number of different methods, from topical application with fine capillaries or brushes to supplementation of liquid and solid food, to physical contact with hormone-soaked objects. In addition, a wide range of doses have been used and sometimes not fully reported, making it difficult to compare between studies. Moreover, mortality was not always reported accurately, or controls were missing. This is further complicated by the range of factors associated with caste determination in ants, including genotype, maternal effects, nutrition, social environment, temperature and combinations thereof (e.g. Gösswald and Bier, 1953; Brian, 1975; Passera, 1980; Helms-Cahan et al., 2002; Anderson et al., 2008; Smith et al., 2008; Penick and Liebig, 2012), and the consequences for the timing of determination. Accordingly, the interpretation of experimental studies on the role of JH in ant queen–worker caste development is not straightforward.

In *Cataglyphis mauritanica* and *Pogonomyrmex barbatus*× *rugosus*, two species with a genetic component to caste determination (Helms-Cahan et al., 2002; Julian et al., 2002), methoprene treatment of eggs (*C. mauritanica*, Kuhn et al., 2018) but not queens (*P. barbatus*× *rugosus*, Helms-Cahan et al., 2011) increased production of queens with worker genotypes. Colonies with treated *P. barbatus*× *rugosus* queens produced larger workers, but this presumed hormone effect cannot be disentangled from treatment-induced changes to colony size (Helms-Cahan et al., 2011). In *P. pallidula*, only eggs produced by overwintered queens shortly after hibernation can give rise to new queens (Passera, 1980); treating queens and eggs with JH I outside this time period resulted in queen production, whereas treatment of larvae had no

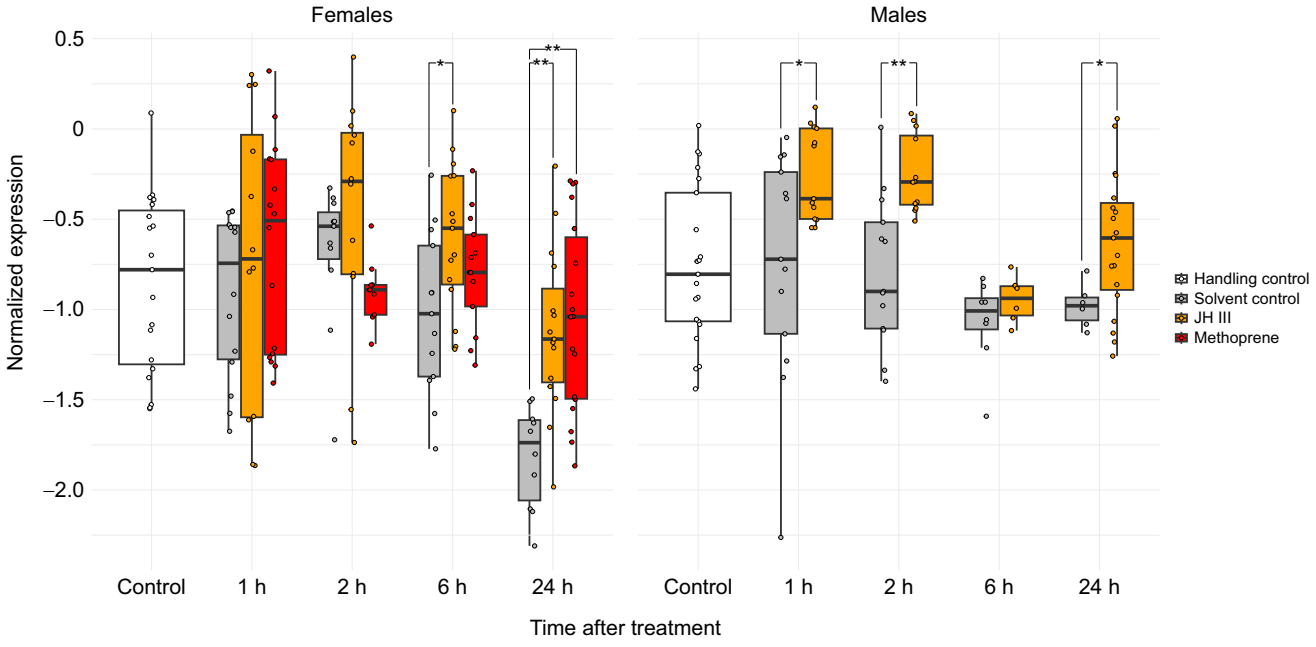

Fig. 3. Timeline of *krüppel-homolog 1* expression in female and male larvae after hormone treatment. Expression in larvae from the three larval instars was compared between treatments, separately for each sex and timepoint, using linear regressions followed by Tukey correction of P-values (*P<0.05, **P<0.01).

effect (Passera and Suzzoni, 1979). Analogous to results on JH, differences in ecdysteroid titers have also been detected in queen- and worker-destined eggs and larvae (Suzzoni et al., 1980, 1983). In another *Pheidole* species, methoprene treatment of queens, eggs and larvae did not result in queen production (Ono, 1982). In *Pogonomyrmex rugosus*, as in *P. pallidula*, only hibernated queens are capable of producing queen-potent eggs (Schwander et al., 2008); here, feeding hibernated colonies with methoprene strongly increased the proportion of queens produced (Libbrecht et al., 2013), although colony productivity and brood mortality were not monitored. In *Solenopsis invicta*, JH-analogue treatment of larvae but not eggs or queens resulted in queen production (Vinson and Robeau, 1974; Robeau and Vinson, 1976; Banks et al., 1978). Finally, in three species with caste determination during larval development, topical treatment with JH analogues resulted in development of more and larger queens in *Myrmica rubra* (Brian, 1974) and increased queen production in *Harpegnathos saltator* (Penick et al., 2012), but feeding with JH III had no effect on queen production in *Camponotus floridanus*, although worker size increased (LeBoeuf et al., 2016).

How does *C. obscurior* fit into this admittedly confusing picture? A previous study found that methoprene treatment of larvae resulted in increased production of winged individuals (Schrempf and Heinze, 2006) but we could not replicate this result. Although we did not systematically test different doses of methoprene, it is unlikely that this discrepancy is caused by a dose effect as the doses used here (~400–40,000 µg g^{-1} body weight depending on the experiment) were within the general range reported in other studies (~60–300,000 µg g^{-1} body weight) and much higher than natural titers of JH reported for insects. For example, whole body extracts of adult female mosquitos contained ~0.008 µg g^{-1} JH (Zhao et al., 2016) and JH titers in queens of the fire ant *Solenopsis invicta* ranged from 0.008 pmol to a maximum of ~0.02 pmol (Brent and Vargo, 2003). The efficacy of doses used in the present study is further supported by the result that JH treatment led to increased expression of the conserved gene *krüppel-homolog 1*. There are several plausible explanations for the conflicting results obtained by Schrempf and Heinze (2006). First, in the present study queen-destined larvae showed a trend toward increased survival compared with workers, so that previous results may have been confounded by differential survival of the two castes. Second, castes differ in size by the second larval instar (Schultner et al., 2023), which may have led to a sampling bias in the previous experiment, so that more larger, queen destined, larvae were included. Third, caste proportions obtained from solvent control treatments (acetone) were not reported in the previous study. In our experiments, treatment of second instar larvae with acetone resulted in a worker-biased caste ratio (Table S1), indicating side-effects of this solvent. In contrast to Schrempf and Heinze (2006), we therefore used ethanol as a solvent for the majority of experiments, also because we found no general negative effects of ethanol on survival (Table 1). Finally, the studies used two different populations of *C. obscurior*, which are known to differ in key traits such as body size, behavior, genome and microbiome (Schrader et al., 2014; Errbii et al., 2021; Ün et al., 2021a,b), as well as the tendency to produce winged males (E.S., unpublished data).

Together with the direct lines of evidence for extreme morphological and molecular differentiation between embryos of the two castes (Wallner, 2021; Oettler et al., 2023 preprint; Schultner et al., 2023), the results of the present study indicate that queen and worker growth and development are highly canalized in *C. obscurior* once caste has been determined. It remains to be tested whether JH treatment of queens leads to the increased production of queen-destined eggs, as in other species with maternal caste determination. In contrast to females, male development appears to have retained a substantial level of plasticity, perhaps because male morph is determined during larval development. The different degrees of canalization in males and females may also be explained by the evolutionary age of male polymorphism, which although basal to *Cardiocondyla* (Oettler et al., 2010), evolved later than queen–worker polyphenism.

Juvenile hormone has long been considered the holy grail in ant queen–worker caste polyphenism, even though no studies have followed up on the original reports in *S. invicta*, *P. pallidula* and *M. rubra*. Wheeler herself acknowledged that after this first intense phase of study, investigations into JH have not been as fruitful as initially hoped (Wheeler, 2003). From the studies summarized above as well as our own results, we nevertheless believe that some conclusions can be drawn. First, the mode and the timing of caste determination are clearly critical factors which can influence JH-responsiveness. Second, JH treatment may override even seemingly 'hard-wired' caste determination modes such as the hybridogenetic system in *Cataglyphis mauritanica*, but the strength of this effect appears to depend on additional individual- and/or colony-level traits. More studies on species with genetic modes of caste determination are needed to validate these findings. Third, any influence experimental JH manipulation may have on caste and body size can be mediated by mortality rates, as these affect brood: worker ratios and colony size, two important social factors in ant development. To avoid this caveat, experiments should ideally be done with standardized colony sizes. Finally, replication of previous experiments coupled with modern molecular methods is needed to evaluate general assumptions about the role of JH in ant caste development, and to help to move this traditional research field into a new era.

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Data availability
All relevant data can be found within the article and its supplementary information.

ECR Spotlight
This article has an associated ECR Spotlight interview with Jeanne Brülhart.

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