1 Crystalline deposits reveal caste identity of late embryos and larvae of the ant

2 *Cardiocondyla obscurior*

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20 Abstract

21 Social insects are interesting models for the study of anticipatory developmental
 22 plasticity because of the striking differentiation into reproductive queens and
 23 functionally sterile workers. A few ant genera, including *Cardiocondyla*, represent the
 24 pinnacle of social evolution in the Hymenoptera, where workers have completely lost
 25 their reproductive organs, minimizing reproductive conflicts between queens and
 26 workers. Here we show that late embryos and larvae of queens of the ant *C. obscurior*
 27 can be identified by the appearance of urate deposits around the forming ovaries.
 28 The discovery of caste-specific urate patterns in *C. obscurior* and three additional
 29 *Cardiocondyla* species will facilitate future studies of developmental plasticity in ants.

Introduction

Alternative developmental trajectories leading to the division of labour between reproductive queens and non-reproductive workers form the basis of superorganismality, thereby permitting one of the major transitions in evolution [1]. Research on caste development in ants has a long tradition (e.g. [2-5], and conceptual discussions are ongoing (e.g. [6,7]. However, how the processes of caste determination and differentiation are regulated at a proximate level is not well understood.

Compared to honeybees, where caste fate is under strict nutritional control, the factors underlying caste fate in ants are more diverse, ranging from genetic to socio-environmental [8]. With this comes variation in the timing of developmental divergence (e.g. [4,9], so that ants exhibit different degrees of “reproductive constraints” [9]. In some species of Ponerine ants, workers retain full reproductive potential, including the ability to mate and store sperm. In the majority of ant species workers have lost the spermatheca but retain more or less functional ovaries capable of producing haploid, male-destined eggs. Finally, workers from 11 genera completely lack ovaries. These obligately sterile workers are an example of an extended phenotype without any direct fitness, representing a highly derived form of superorganismality. The biology of some myrmicine species with fully sterile workers has been studied extensively (*Cardiocondyla obscurior*, *Monomorium pharaonis*, *Pheidole spec.*, *Solenopsis invicta*), but comparably little is known about their development. Even less is known about development in the remaining seven genera with workers lacking reproductive organs (to the best of our knowledge; Myrmicinae: *Wasmannia*, *Tetramorium*, *Pheidologetum*; Dorylinae: *Eciton*; Ponerinae: *Leptogenys*, *Hypoponera*, *Anochetus*).

Across the range of reproductive constraints, a diverse set of signals spanning nature and nurture is likely to be involved in caste-specific development. Together with the facts that ant larval mobility is variable, ant brood is reared in piles, and brood is often

relocated and can serve functional roles in the colony [10], this has rendered the study of ant development a more complex problem compared to *Drosophila* research. In particular, it has been difficult to study caste-specific developmental trajectories because it is not possible to distinguish worker-destined larvae from queen-destined and male-destined larvae (e.g. [11]). Here, we close this gap for the ant *Cardiocondyla obscurior* by showing that queen- and worker-destined embryos and larvae can be visually distinguished by crystalline deposits surrounding the developing ovaries of queen-destined larvae. This discovery will facilitate the study of caste determination and differentiation at the extreme end of the superorganismality spectrum, thus bringing us closer to a general understanding of the mechanisms facilitating the evolution of social insects.

Methods

Ants

C. obscurior is a cosmopolitan tramp ant [12], with a very streamlined genome (~193MB, [13]), the smallest ant genome known to date. Adult queens and workers differ in size and morphology and workers lack ovaries. Larvae develop via three larval instars which can be distinguished by the shape of the body and the degree of sclerotization of the mandibles [14]. The ants used in this study were all from the Old World lineage [13], maintained in the lab since 2010. Stock colonies were kept in a climate chamber under a 12h/12h and 22°C/26°C night/day cycle at 70% humidity. Experimental colonies were kept in round plaster-bottom nests with nest indentations covered by dark foil under the same conditions. All colonies were provided with water and fed three times a week with honey and pieces of cockroaches and fruit flies.

White crystalline spots

All larvae of *C. obscurior* exhibit white spots. After producing semi-thin sections (see below), we used a polarization filter that revealed a crystalline reflection. These crystalline structures are common in insect larvae and have been described as urate crystals (see discussion). Hence, because it is a parsimonious explanation we use “urate deposits” in the following when we refer to the white spots, even though we are aware that this may not be correct and requires future verification.

Urate deposit localization

We characterized urate deposits in eggs and first instar (L1), second instar (L2) and third instar (L3) larvae as unpaired (= worker-destined) or paired (= queen-destined) by visually inspecting brood from stock colonies under a stereomicroscope. For better detection of the patterns, eggs and L1 larvae were submerged in a dissection dish containing PBT (0.3 %), after which they were mounted on a microscope slide and sealed with nail polish. From each development stage, we selected and photographed one representative individual with a paired pattern and one individual with an unpaired pattern using a stereomicroscope connected to a camera (Keyence VHX 500FD, Neu-Isenburg, Germany).

We additionally characterized urate patterns of third instar larvae from seven *Cardiocondyla* species available in the lab. We further examined brood of six species from four subfamilies available as live colonies. Lastly, we accessed Alex Wild’s photo library for a broader overview of species (<https://www.alexanderwild.com/Ants/Natural-History/Metamorphosis-Ant-Brood/>).

Caste fate and survival according to urate deposits

We tracked development of all stages to confirm that urate localization patterns are associated with caste in *C. obscurior*. Brood was sampled from several stock colonies and sorted by development stage and urate pattern as described above. Eggs and L1

larvae were transferred to filter paper to remove excessive buffer. Eggs were then transferred in groups of ~15 to experimental colonies containing 10 workers (eggs: paired=192, unpaired=165). L1 larvae were transferred to experimental colonies containing 10 workers; two colonies were setup with 17 queen-destined larvae each, and two colonies with 19 and 4 for worker-destined larvae, respectively (L1: paired=34, unpaired=23). L2 and L3 larvae were transferred in groups of ten to experimental colonies containing 10-12 workers (L2: paired=50, unpaired=40, L3: paired=50, unpaired=40). Experimental colonies were monitored three times per week and, when necessary, workers added from the corresponding stock colonies to standardize worker number. All pupae were counted and classified according to female caste until no more brood remained. From these data, we calculated average survival until pupation and caste ratios. Survival of castes and accuracy of caste prediction were tested with Fisher exact tests (fisher.test in R).

We tracked development of L3 larvae from six additional *Cardiocondyla* species, *C. wroughtonii*, *C. argyrotricha*, *C. thoracica*, *C. minutior*, *C. nuda*, *C. venustula*, spanning the phylogeny with exception of the palearctic clade [15], to validate that urate deposit patterns accurately predict caste.

Larval histology

L3 larvae were collected from stock colonies of *C. obscurior* and sorted according to their urate patterns. Sorted larvae were transferred into a fixation solution consisting of 25% glutaraldehyde (GAH) in cacodylate buffer [(50 mM cacodylic acid, pH 7.3) containing 150 mM Sucrose] (GAH : cacodylate buffer = 1 : 12,5) and kept overnight at 11 °C. Samples were then rinsed in cacodylate buffer on ice, and fixated in 4% osmium tetroxide in cacodylate buffer. After fixation, larvae were washed in cacodylate buffer, dehydrated in a graded ethanol series and embedded in Epon. Transversal semithin sections of 1 µm were cut and stained with methylene blue and

Azur II (Richardson's stain). Semi-thin sections were scanned with a Zeiss Primo Star microscope and imaged with a Moticam 580 digital microscope camera.

Ovary development

C. obscurior queen L2 and L3 larvae were dissected in PBT 0.3% (PBS 1x + Triton 0.3%) by cutting below the midgut with a micro scissor. The posterior part of the larvae was cleared of excess fat tissue. The larval ovaries were placed in an ice-cooled well filled with PBT. We chose two different stages of queen pupae based on their development. Early-stage pupae were identified as unsclerotized white pupae with unpigmented ommatidia and ocelli. Mid-stage pupae exhibit pigmented ommatidia and ocelli. Ovaries from queen pupae and adults were obtained by carefully pulling on the last tergite of the abdomen with forceps, which removes the entire reproductive apparatus. Larval, pupal and adult ovaries were fixated in 4% paraformaldehyde diluted in PBS for 20 min at room temperature. The fixated ovaries were then washed three times with PBT for 15 min on a tumbler. After washing, the ovaries were processed for staining.

Vasa protein was stained with a rat anti-vasa antibody and actin filaments were visualized using a rabbit anti-actin antibody. Both antibodies were generated from *Drosophila* [9]. Cell nuclei were stained using DAPI. The primary antibodies were diluted 1:200 in PBT 0.3% with 5% normal goat serum overnight at room temperature on a tumbler. To remove the primary antibody, the larval and pupal ovaries were washed three times with PBT for 15 min. The secondary antibodies goat anti-rat and goat anti-rabbit were used to visualize the rat anti-vasa and rabbit anti-actin antibodies, both diluted 1:200 in PBT. After 2 h of incubation at room temperature, the ovaries were again washed three times with PBT for 15 min each. Finally, the larval, pupal and adult ovaries were washed with PBT containing DAPI (1:10000). Ovaries were then mounted in Vectashield on a microscope slide and sealed with nail polish.

Images for the antibody staining were obtained using a Leica SP8 confocal microscope under 40x and 63x objective lenses. Complete confocal series of ovarioles of early- and mid-staged queen pupae were processed using the FIJI plugin TrakEM2 [16] for 3D rendering and analysis.

Nanos and *vasa* expression was measured in queens and workers from all larval stages. L1 and L2 larvae were collected and transferred to individual 1.5 ml tubes (L1: n=5 queen larvae, n=7 worker larvae; L2: n=5 queen larvae, n=5 worker larvae). L3 larvae were cut beneath the midgut at the posterior end (containing the developing ovaries in queen larvae) and transferred to individual tubes (L3: n=5 queen larvae, n=4 worker larvae). All samples were immediately frozen in liquid nitrogen and stored at -75°C prior to RNA extraction. Total RNA was extracted using the ReliaPrep RNA Cell Miniprep Kit (Promega) and RNA concentrations measured using the Qubit RNA HS Assay Kit (Invitrogen). RNA concentrations were standardized to 2.5 ng and RNA reverse-transcribed to cDNA using the iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories). Expression of the gene *nanos* was quantified with qPCR with the primer pair *nos_for/nos_rev* and normalized with two housekeeping genes (Y45F10D_JO1_for/Y45F10D_JO1_rev [17]) and *Actin_JO1_for/Actin_JO1_rev*; see Table S1 for primer sequences). All qPCR reactions were performed in triplicates and specificity of reactions confirmed with manual melt curve inspection. Relative target gene expression was calculated as $2^{-\Delta C_q}$ following [18], using the geometric mean of the two housekeeping genes for normalization.

Results

Queen and worker-destined eggs and larvae of *C. obscurior* can be distinguished, by what appear to be urate crystals. In late embryos (Figure 1) and the three larval stages (Figure 2, Figure S1), urate deposit patterns associated with queen caste are visible. Queen patterns appear either as a string of pearls or are snail shell-like (Figure S2).

Handling L1 larvae results in high mortality, but L2 and L3 individuals can be attributed to the two castes with high precision (Table 1).

Transversal semithin sections of L3 larvae revealed that worker larvae lack ovaries and have urate deposits which are distributed randomly throughout the caudal end (Figure 3 A). These cluster in small aggregates (10-20 μm diameter) in proximity to trophocytes (Figure 3 C, E). In queen larvae, urate crystals form voluminous aggregations surrounding the developing ovaries, which contain several dozens of developing oocytes (Figure 3 B, D, F). In both castes urate comes in the shape of translucent birefringent spherocrystals.

Urate deposit patterns allowed for selection of queen-destined L2 and L3 larvae, making it efficient to reconstruct ovarian development. In L2 larvae the developing ovaries are visible as a bilateral cluster of germ cells located at the posterior end of the last abdominal segments (Figure 4 A-D). The ovaries are in close proximity to the caudal end of the central nervous system (Figure 4 D, CNS). The cluster of cells in L2 larvae then differentiate into three ovarioles per ovary in L3 larvae (Figure 4 E-H), with the tips extending in an anterior-apical orientation (Figure S8). Disc-shaped cells can be found which stack in a medial-lateral orientation (Figure 4 H, asterisk). These cells are likely cap cells [19], which separate the germarium from the terminal filament [20].

In pupae, the ovaries are completely differentiated, with fully extended terminal filaments (Figure 4 I-L). The germ cells in the germarium have differentiated into cystocytes, which will later give rise to the oocyte with the associated nurse cells (Figure 4 L) [21]. After reaching the adult stage, the fully matured ovarioles have egg chambers, containing oocytes with their corresponding nurse cells (Figure 4 M-P). The oocytes are surrounded by follicle cells, and these egg chambers are in different stages of maturation (Figure 4 P). Reconstruction of the cystocytes inside the ovarioles of early- and mid-staged queen pupae showed that the developing cells increase significantly in size during pupation (Linear regression, $R^2=0.9036$, $p<0.001$) and that cell size is highly variable (Figure S3).

The expression of the germline marker *nanos* was higher in L2 and L3 queen larvae compared to worker larvae (Figure S4, L2: Mann-Whitney-U-test: $W=24$, $p=0.016$; L3: $W=20$, $p=0.016$, Figure S4), but not in L1 larvae ($W=25$, $p=0.27$). *nanos* expression increased over the course of development in queens, matching the developmental state of the ovaries. The variation in *nanos* expression is conspicuously high in L2 queen larvae. In contrast, *vasa* expression was significantly higher in L1 ($W=33$, $p=0.010$) and L3 queen larvae ($W=20$, $p=0.016$), but not in L2 larvae ($W=17$, $p=0.421$), confirming that sampling based on urate patterns was successful, despite failure to show this for L1 larvae in rearing experiments.

We found queen-like urate patterns in larvae of three additional *Cardiocondyla* species (*C. venustula*, *C. wroughtonii* and *C. nuda*) (Figure S5, Table S2). In the remaining four species (*C. thoracica*, *C. elegans*, *C. argyrotricha* and *C. minutior*) we did not find obvious caste-specific patterns (Figure S6, Table S2).

39 of 41 additional species representing 30 genera from seven ant subfamilies had larvae with obvious urate deposits (Table S3; Figure S7), but no species exhibited paired urate deposits resembling the pattern observed in *Cardiocondyla* queen larvae.

Discussion

“Auch ein blindes Huhn findet mal ein Korn” (German saying)

Cardiocondyla is unique among the Formicidae due to the evolution of wingless fighter males and the repeated loss of the winged male morph across the genus [15]. Because colonies of *C. obscurior* can be easily reared and manipulated, the species has been used to address questions in diverse biological contexts [22,23], ranging from genome evolution [13,24], to social behavior [25], aging [26,27], symbiosis [28,29], and phenotypic plasticity [17,30,31]. In this respect, *C. obscurior* has been considered a “gold mine” (E. Abouheif pers. com.). Now, as is often the case in

mining, sheer luck has led to the discovery that queen and worker embryos and larvae in some *Cardiocondyla* species can be distinguished using the localization of urate deposits. We could not find a similar pattern in other ant genera, but 39 species from 30 genera is by far not representative of the vast diversity of ants. We hope that our discovery inspires others to take a closer look at the brood of their favorite species.

In insects, urate is typically deposited in specialized cells – so-called urocytes [32] – which together with trophocytes [32] and oenocytes [33] represent the three major cell types in the fat body. Urocytes store urates either in vacuoles or as circular, birefringent spherocrystals [32], and can occupy a large fraction of the fat body, for instance in the termite *Mastotermes darwiniensis* [32]. In the termite *Reticulitermes flavipes*, uric acid is recycled by gut bacteria as a nitrogen source [34], and urate deposits have been linked to oocyte development [35]. In *Pachycondyla* ants, urocytes specialize in the storage of nutrients and excretion products [36] and a composition assay in *Melipona quadrifasciata* bees identified Na, Ca, Mg, P as main elements in urocytes, as well as traces of Zn, Mn, and K [33]. In *C. obscurior* we found circular, birefringent spherocrystals but no cell nuclei, indicating that urate is deposited directly. Worker larvae exhibited small, randomly distributed urate clusters which were found closely associated with what appeared to be trophocytes rather than urocytes. These smaller clusters may originate as a by-product of metabolic processes in trophocytes. Queen larvae showed dense aggregations of urate surrounding the developing ovaries. These large urate deposits may constitute an energy reservoir for cellular growth, a process that is energy-demanding since cells in and around ovaries proliferate at rapid rates.

Caste-specific urate patterns only occurred in some *Cardiocondyla* species and in none of the species representing other ant genera. At the moment, we do not know why this is the case. It is unlikely that the presence of the bacterial symbiont *Cand. Westeberhardia cardiocondylae* is directly linked, although in principle it makes for a promising candidate. This endosymbiont with a highly eroded genome resides in

bacteriomes associated with the gut and, prominently, with the ovaries in queens, indicating a nutritional link. However, the same urate patterns occur in a *C. obscurior* lineage that is naturally free of *Cand. Westeberhardia* [29], as well as in larvae which have been experimentally cleared of their bacterial symbionts with antibiotic treatment [28]. There is also no obvious phylogenetic signal. *C. obscurior* and its sister species *C. wroughtonii* both exhibit caste-specific patterns but cluster together in clade A with *C. thoracica* and *C. argyrotricha*, both of which do not show the pattern. In clade B, *C. nuda* and *C. venustula*, which exhibit the caste-specific patterns, cluster together with *C. minutior* [15]. A welcome additional coincidence is that all four species with the distinct queen pattern have two alternative male morphs [15] and can be kept in the lab with minimum effort, making the genus perfect for comparative studies of female and male diphenic development.

Urate deposit patterns allowed us to reconstruct ovarian development in *C. obscurior*. The female reproductive apparatus is conserved in insects, and consists of two ovaries, the oviduct, the uterus, accessory glands and the spermatheca. Large variation can be found in the number of ovarioles making up each ovary, ranging from 1 in a dung beetle, 15-20 in the fruit fly *Drosophila melanogaster* [19], ~200 in honey bees [37] to more than 1000 in some termite and ant species [38]. Six ovarioles in *C. obscurior* and *Cardiocondyla nuda* queens [39] mark the lower end in ants, thus allowing detailed study of ovary development in a simple model. In *C. obscurior*, the ovaries develop from a cluster showing early differentiation in L2 larvae to ovaries with differentiated ovarioles in L3 larvae. Nevertheless, the size of urate deposits in L2 larvae is similar to that in L3 larvae. Future studies investigating development prior to the L2 stage will reveal when exactly queen and worker phenotypes diverge, and uncover how urate deposits are related to ovary formation.

Germline stem cells (GSCs) are typically located in the germarium at the tip of the ovarioles, close to the terminal filament cells ([9,20]. GSCs continuously renew themselves and produce new germ cells. They then proliferate and produce

differentiated cystoblasts, which divide synchronously to form cystocytes [21]. These cystocytes are found in the germarium of pupal ovarioles in *C. obscurior*, with some cells larger than others. Cystocytes go on to differentiate into oocytes with their corresponding nurse cells. We suspect that the largest cells are pre-determined oocytes in the pupal germarium, which allow to initiate oocyte maturation soon after hatching. *Cardiocondyla* queens mate inside their natal nest within days of reaching adulthood and can almost immediately begin with the production of offspring [27].

The discovery of caste-specific patterns has important implications for understanding both the biology of *C. obscurior* and the general mechanisms underlying caste development in ants. At the life history level, it is now possible to study questions revolving around the phenotypes of developing queens and workers. For example, do the larval phenotypes differ in morphology, behavior or cuticular chemistry? We can also test whether workers discriminate between queen- and worker-destined brood, and if larvae are treated differently depending on caste. In the future, it should even be possible to track individual larvae without the need for tagging by using their unique patterns for individual recognition, similar to the way fins are used to study humpback whales [40]. Creativity limits possibilities now, not vice versa.

Because caste-specific urate patterns are already visible in late embryos, we are also finally able to study caste development beyond differentiated features such as wing discs in late larvae [41]. The establishment of *nanos* and *vasa* as reliable expression markers further allows for verification of individual caste identity in molecular studies. *C. obscurior* queens produce increasingly queen-biased caste ratios with age [27], facilitating efficient sampling of queen-destined embryos, and opening numerous avenues for the study of the mechanisms involved in ant caste determination and differentiation, and in social insect polyphenism in general. It has already been shown that ant and honeybee development shares some features, with an overlapping set of genes involved in growth (e.g. TOR, Insulin-like) [42]) and juvenile hormone synthesis and degradation [43] playing a role in developmental regulation [8,30]. Upstream, the

importance of transcription factors involved with the sex differentiation cascade also finds support in ants [17,44], and honeybees [45,46]. Recently, we postulated that a major evolutionary transition, the evolution of sex, has facilitated another major transition, the evolution of eusociality, via co-option of the same developmental switch mechanism [17]. Now we can test this hypothesis, bringing us one step closer to a conceptual understanding of how social insect polyphenism evolved.

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502

Table 1: Urate deposit patterns predict caste in the ant *Cardiocondyla obscurior*. Survival differences between castes and accuracy of caste prediction were tested using Fisher's exact tests.

Development stage	Predicted caste	n	Survival			Produced caste		
			Proportion (%)	Differences between castes		Proportion (%)	Accuracy of caste prediction	
				Odds ratio	p		Odds ratio	p
egg	queen	165	24.9 (41/165)	1.276	0.342	78 (32/41)	5.398	<0.001
	worker	192	29.7 (57/192)			42.1 (24/57)		
1st instar larva	queen	34	5.9 (2/34)	1.512	1	0 (0/2)	-	-
	worker	23	8.7 (2/23)			100 (2/2)		
2nd instar larva	queen	50	62 (31/50)	0.334	0.019	90.3 (28/31)	97.129	<0.001
	worker	40	35 (14/40)			92.9 (13/14)		
3rd instar larva	queen	50	60 (30/50)	0.439	0.062	100 (30/30)	infinite	<0.001
	worker	40	42.5 (17/40)			100 (17/17)		

Figure legends

Figure 1: Urate deposit patterns distinguish queen- and worker-destined larvae in the ant *Cardiocondyla obscurior*. Stereomicroscope images of worker-destined larval instars (A, C, E) and queen-destined larval instars (B, D, F). Ur = urate.

Figure 2: Urate localization in queen- and worker-destined embryos in the ant *Cardiocondyla obscurior*. Worker-destined embryos show no urate (ur) (A), while queen-destined embryos have urate localized in the last segments of their germ band (gb) (B). The first segments of the embryo are clearly separated into the head and the three gnathal segments: mandibular (md), maxillary (mx) and labial (lb).

Figure 3: Histological sections of *Cardiocondyla obscurior* larvae. (A) Transversal section of a worker-destined L3 larva. In workers ovaries are missing. (B) Queen-destined larvae show paired ovaries (ov) close to the midgut (mid). (C) In worker-destined larvae urate (ur) accumulates in small randomly distributed deposits. (D) Ovaries in queen-destined larvae are surrounded by large urate deposits. (E) Small

urate deposits aggregate close to trophocytes (t). (F) Urate encloses the developing ovary in queen larvae. Tf = terminal filament.

Figure 4: Ovarian reconstruction in queens of *Cardiocondyla obscurior*. (A-D) Early gonadal formation in second instar larva. DAPI is used to show the cell nuclei. Vasa stains against germ cells and actin visualizes the cytoskeleton. (A) DAPI staining, (B) Vasa staining, (C) Actin staining, and (D) Merged image with a visible central nervous system (CNS). (E-H) Ovarioles (ov) of a third instar queen larva. (E) DAPI, (F), Vasa, (G) Actin and (H) Merge. The asterisk (*) shows cap cells. (I-L) Ovary of a queen pupa with its six ovarioles. (I) DAPI, (J), Vasa, (K) Actin and (L) Merge. The germ cells have differentiated into cystocytes in this stage, inhabiting the caudal end of the ovariole. Tf = terminal filament. (M-P) Mature ovarioles in adult queens. (M) DAPI, (N) Vasa, (O) Actin and (P) Merge showing the oocyte (oo) with its nurse cells (nc). The oocyte is encircled by follicle cells (fc).

Supplementary Figure legends

Supplementary Figure 1: Dorsal and ventral view of urate patterns in worker- and queen-destined larvae in the ant *C. obscurior*. Stereomicroscope images of worker-destined larval instars (A, C & E) and queen-destined larval instars (B, D & F). (A, B) show larvae in ventral orientation, (C-F) show larvae in ventral orientation. Urate deposits are visible as bilateral symmetrical clusters in queen-destined larvae.

Supplementary Figure 2: Types of urate patterns in queen-destined larvae. (A, B) First instar larva with string of pearl-like urate deposits. (C, D) First instar larva with snail shell-like urate deposits.

Supplementary Figure 3: Images of queen pupae. (A) Early-stage queen pupa with unpigmented ommatidia and ocelli. (B) Mid stage queen pupa with pigmented ommatidia and ocelli. (C) Example of a 3D rendered ovariole with TrakEM2. Data generated from the reconstruction was used for data analysis. Colors represent

individual cells. (D) Cell volume to cell surface in *C. obscurior* queen pupae. The plot illustrates the different sizes of cells in developing cystocytes of queen ovarioles from two pupal stages.

Supplementary Figure 4: Caste-specific expression of the germline marker *nanos* in the ant *C. obscurior*. Normalized gene expression of the germline marker in queens and workers in all three larval stages. Expression of *nanos* is significantly higher in L2 and L3 queen larvae compared to worker larvae. * $P < 0.05$, Mann-Whitney-U-test.

Supplementary Figure 5: Urate patterns distinguish queen and worker-destined larvae in various *Cardiocondyla* species. Stereomicroscope images of queen-destined larval instars (A, B, C) and worker-destined larval instars (D, E, F). (A) Queen larva of *C. venustula* (B) Queen larva of *C. wroughtonii*. (C) Queen larva of *C. nuda*. (D) Worker larva of *C. venustula*. (E) Worker larva of *C. wroughtonii*. (F) Worker larva of *C. nuda*. Ur = urate.

Supplementary Figure 6: Urate patterns in *Cardiocondyla* species without paired urate deposits. Stereomicroscope images of larval instars. (A) *C. argyrotricha*. (B) *C. minutior*. (C) *C. thoracica*. (D) *C. elegans*.

Supplementary Figure 7: Urate localization patterns in seven species from four ant subfamilies. Stereomicroscope images of larval instars. (A) *Camponotus mus* (Formicinae). (B) *Formica rufibarbis* (Formicinae). (C) *Linepithema humile* (Dolichoderinae). *Platythyrea punctata* (Ponerinae) (dorsal view). (E) *Wasmannia auropunctata* (Myrmicinae). (F) *Temnothorax* sp. (Myrmicinae). Both representatives of the Formicinae are lacking visible urate deposits.

Supplementary Figure 8: Schematic illustration of postembryonic ovarian development in the ant *C. obscurior*. The sketched images represent the 2nd instar (A), 3rd instar (B) and pupal (C) stage of queens. In the 3rd instar the lateral oviduct (red) is already distinct. The green coloration represents the germ cells as seen in Figure 4.

Figure 1

Figure 2

Figure 3

Figure 4