Maternal control over caste allocation in the ant *Cardiocondyla obscurior*

Eva Schultner[1*]

Tobias Wallner[1]

Benjamin Dofka[1]

Jeanne Bruelhart[1]

Jürgen Heinze[1]

Dalial Freitak[2]

Tamara Pokorny[1]

Jan Oettler[1*]

[1]Zoology/Evolutionary Biology, University of Regensburg, Universitätsstrasse 31, 93053 Regensburg, Germany

[2] Institute for Biology, University of Graz, Universitätsplatz 2, 8010 Graz, Austria

*corresponding authors

ORCIDs:

Eva Schultner: 0000-0002-5069-9732

Tobias Wallner: 0000-0001-9135-6456

Tamara Pokorny: 0000-0002-8183-9638

Dalial Freitak: 0000-0001-8574-0531

Jan Oettler: 0000-0002-8539-6029

Keywords:

Social insects, caste determination, development, eggs, larvae, discrimination behavior

Author contributions:

ES and JO supervised the study and wrote the manuscript. ES analyzed and visualized the data. TW discovered the crystalline deposits and together with BD contributed to the rearing and behavioral experiments. JB contributed morphological data, DF contributed protein data, TP contributed cuticular hydrocarbon data. All authors read and approved the final version of the manuscript.

Abstract

Ant queens and workers commonly engage in conflict over reproductive allocation because they have different fitness optima. How such conflicts are resolved depends on the power that each party of interest holds. Here, we show that queens have control over caste allocation in the ant *Cardiocondyla obscurior*. With the discovery of crystalline deposits that allow the identification of queens and workers across the entire course of development, we demonstrate that caste fate is irreversibly determined in the egg stage. Comparison of queen and worker-destined eggs and larvae revealed size and weight differences in late development, but no discernible differences in traits that may be used in social interactions, including hair morphology and cuticular odors. In line with this lack of caste-specific traits, adult workers treated developing queens and workers indiscriminately, even in the presence of a fertile queen. Together with previous studies showing queen control over sex allocation, these results provide evidence that conflict over reproductive allocation is absent in this ant.

Introduction

Ant queens and workers are fixed in irreversible, evolutionarily co-dependent roles, analogous to the germ line and soma of multicellular organisms [1–3]. However,

compared to multicellular organisms, ant colonies can show relatively high levels of within-unit conflict. This is because they are made up of non-clonal individuals, with factors such as polygyny (multiple reproducing queens per colony) and polyandry (multiple mating by queens) causing variation in relatedness among nestmates [4,5]. As a result, colony members can differ in their fitness optima regarding reproductive allocation, creating the potential for conflict [6–8].

Whether potential conflict leads to actual conflict depends on the power each party holds, in particular over reproduction and access to information [9–11]. Adult reproductive power can be classified along a range of worker reproductive constraints [12–15]: in species where workers retain fully or partly functional reproductive organs (most ant genera), adult females can engage in conflict over reproductive dominance or male parentage by participating in production of female and male offspring [16–18]. In the few ant genera in which workers have lost reproductive organs entirely, they cannot participate in reproduction directly. Nonetheless, because workers are responsible for brood care, even in these species they can influence reproductive allocation by manipulating the sex and caste ratios of brood, e.g by preferentially rearing females into queens or by culling male or queen-destined brood [19–22]. Across the range of reproductive constraints, the fitness interests of developing individuals also play an important role, in particular in the context of queen-worker caste determination [21,23,24]. In all conflict scenarios, access to information about brood maternity, sex and caste can be decisive, placing developing individuals at the center of within-colony conflict [25,26].

Cardiocondyla ants represent the extreme end of the range of reproductive constraints because workers lack reproductive organs, precluding any direct conflict over reproduction. In *Cardiocondyla obscurior*, workers further refrain from manipulating brood sex ratios, giving queens control over colony-level sex allocation [27,28]. Recently, we found that in single queen colonies of this ant, production of new queens increases as the resident queen ages, irrespective of absolute queen

fitness, queen lifespan or colony size [29]. This suggests that queens also control caste allocation, and points towards relaxed selection on potentially costly traits related to caste allocation conflict.

Here, we explore this idea by providing a description of the phenotypes of developing *C. obscurior* ant queens and workers, made possible by the discovery of crystalline deposits that distinguish castes from the egg stage onwards. We focus on traits that are predicted to differ between castes and could transmit potential information about caste in a social context, and test whether workers discriminate between developing queens and workers. Our results support the hypothesis that queens control primary caste ratios in eggs and workers refrain from manipulating secondary caste ratios of brood, giving queens complete control over caste allocation in this ant.

Results and Discussion

Identifying female caste in developing ants

"Auch ein blindes Huhn findet mal ein Korn"

Twenty years after the first *C. obscurior* colony was brought to the laboratory in Regensburg, we discovered that queen and worker-destined eggs and larvae can be distinguished by the caste-specific distribution of white spots in the posterior end (Figures 1,2). To our knowledge, this is the first description of a discrete trait that can be used to identify female caste over the entire course of ant development. Semi-thin sections of third instar larvae viewed through a polarization filter revealed these spots have a crystalline reflection. Queens exhibit a pair of clumped crystalline deposits on the dorsal side, which become larger and more distinct as development progresses (Figures 1,2, Figure S1). In late-instar queen larvae, crystalline deposits form large aggregations around the developing ovaries, which already contain immature oocytes (Figure 3). Workers do not have crystalline deposits in the egg stage (Figure 2), and small, randomly distributed deposits in larval stages (Figures 1, 3). In both

106 castes, crystalline deposits may consist of urate crystals, which are usually stored in
107 specialized cells, the urocytes, in the insect fat body [30], and which have been linked
108 to nutrient storage in ant larvae [31] and oocyte development in termite reproductives
109 [32].

110 Crystalline deposit patterns can be used to identify individuals by caste with high
111 accuracy, and workers successfully reared both castes at comparable rates in colony
112 fragments without queens (Table 1). This demonstrates that caste fate is fixed in the
113 embryo, likely under physiological control of the queen, and provides a first indication
114 that workers do not discriminate between developing queens and workers.

115 Similar crystalline deposit patterns that reliably predict caste were found in third instar
116 larvae of *C. nuda*, *C. venustula* as well as *C. wroughtonii,* the sister species to *C.*
117 *obscurior* (Figure S2, Table S1). No caste-specific patterns were found in *C.*
118 *"argyrotricha"* (provisional name of a recognized morphospecies to be described by
119 B. Seifert), *C. elegans*, *C. emeryi*, *C. minutior* and *C. thoracica* (Figure S3, Table S1),
120 or in any of the other 42 screened ant species (Figure S4, Table S2). This patchy
121 pattern does not follow the phylogenetic relationships within the *Cardiocondyla*
122 genus [33]. Caste-specific patterns are also not linked to the presence of the main
123 bacterial symbiont *Cand.* Westeberhardia cardiocondylae, an endosymbiont with a
124 highly reduced genome that resides in gut-associated bacteriomes, as well as in
125 ovaries in queens [34]. *Cardiocondyla minutior* carries the symbiont [35] but does not
126 display caste-specific patterns, while a *C. obscurior* lineage that is naturally free of
127 *Cand.* Westeberhardia [34,36] and has been maintained in the lab since 2011 shows
128 the patterns (Schultner, pers. obs.). This does not rule out a connection between
129 symbiont but indicates that a potential link must entail more than mere symbiont
130 presence. Future studies will uncover the metabolic processes from which the
131 deposits stem and reveal their function.

132 **Phenotypes of developing queens and workers**

133 General morphologies of ant larvae have been extensively described, e.g. [37–40],
134 and non-invasive identification of larval sex has been demonstrated previously [41].
135 Traits that distinguish late-stage worker from sexual larvae (males, queens) have been
136 discovered in six ant species from three subfamilies; these are largely continuous
137 measures, including body size (*Aphaenogaster senilis* [42]; *Harpegnathos saltator*
138 [43]; *Myrmica rubra* [44]), cuticle "shininess" (*Linepithema humile*, [45]), hair density
139 (*Monomorium pharaonis* [46]), and hair morphology *(Acromyrmex echinatior* [47]).
140 Apart from the crystalline deposits, *C. obscurior* queen and worker larvae appeared
141 similar in their morphology, and neither the density nor the morphology of hairs
142 differed between castes in any of the three instars (Figure S5). There was also no
143 difference in size between the castes during early development (Figure 4, eggs:
144 Linear mixed model, length: χ^2=0.123, df=1, p=0.726; width: χ^2=0.007, df=1,
145 p=0.934; L1 larvae: ANOVA, head width: $F_{67,1}$=3.009, p=0.088; body length:
146 $F_{52,1}$=0.366, p=0.548). However, as expected in an ant with morphologically distinct
147 queen and worker castes [48], queen larvae grew larger than worker larvae as
148 development progressed (Figure 4, L2 larvae: ANOVA, head width: $F_{60,1}$=3.124,
149 p=0.088; body length: $F_{60,1}$=14.542, p<0.001; L3 larvae: ANOVA, head width:
150 $F_{60,1}$=17.198, p<0.001; body length: $F_{60,1}$=11.754, p<0.01). Third instar queen larvae
151 were also heavier than worker larvae (Figure 5A, L2 larvae: ANOVA, $F_{10,1}$=3.547,
152 p=0.096; L3 larvae: ANOVA, $F_{10,1}$=98.208, p<0.001) but there was no difference in
153 protein content between the two larval castes (Figure 5B, L2 larvae: ANOVA,
154 $F_{41,1}$=0.133, p=0.717; L3 larvae: ANOVA, $F_{49,1}$= 0.876, p=0.354).

155 Chemical analyses of cuticular odors of queen and worker third instar larvae and
156 adults of *C. obscurior* detected 32 compounds (Table 2). The profiles of adult queens
157 and workers were slightly more complex than those of larvae (adults: >30 compounds
158 including trace amounts below 0.5%; larvae: 24 compounds). Larval profiles were
159 dominated by n-alkanes, with pentacosane and heptacosane constituting over 65%
160 of the total profile. These two compounds were also present in high relative amounts

161 in adults, together with unsaturated and methyl-branched hydrocarbons. Principal
162 component analysis clearly separated samples by development stage on PC1, apart
163 from two adult samples, which clustered with larva samples for unknown reasons
164 (Figure 6, Table S2). Differences between development stages were confirmed by
165 analysis of similarities (adult vs. larva profiles: ANOSIM, R=0.628, p<0.001). PC2
166 separated adult castes, with a single worker sample clustering with queens (Figure 6,
167 Table S2, ANOSIM, R=0.548, p<0.001). In contrast, the profiles of queen larvae and
168 worker larvae did not differ (Figure 6, Table S2, ANOSIM, R=-0.059, p=0.651). A
169 similar lack of caste-specific larval odors has been demonstrated in late-stage larvae
170 of the ant *Aphaenogaster senilis* [42], while in *Harpegnathos saltator*, odor profiles of
171 queen-destined larvae were distinct from those of worker-destined larvae, allowing
172 workers to identify larvae by caste [43]. Ant larvae are often considered chemically
173 insignificant because they are easily accepted across colonies, populations and even
174 species (reviewed in [26]. At the same time, brood odors can play an important role
175 in queen fertility signaling within colonies, for example when eggs and larvae carry
176 odors that match those of queens [49,50]. Because of the relative difficulty of
177 distinguishing ant brood by developmental stage, sex and caste, detailed data remain
178 limited, and more studies are needed to understand the function of brood odors.

179 **No sign of caste conflict involving brood**

180 In line with the absence of caste-specific morphological and chemical cues, adult
181 workers did not adjust their behavior to the caste of encountered larvae in any of the
182 five tested scenarios. Workers were similarly attracted to queen and worker larvae
183 (Figure S6, number of contacts: ANOVA, $F_{44,1}$=3.193, p=0.077; duration of contacts:
184 Mann-Whitney-U-test, W=1184, p=0.072) and did not preferentially retrieve larvae of
185 either caste (queen larvae retrieved: 59% (40/67), worker larvae retrieved: 40%
186 (27/67), Chi-squared test, χ^2=2.522, df=1, p=0.113). Observations of feeding
187 behavior revealed no preference for either caste, with 65% (13/20) of worker larvae
188 and 59% (10/17) of queen larvae containing fluorescent dye in their gut two hours

189 after starved colonies composed of larvae, workers and queens were fed with
190 fluorescent honey (Fisher's exact test, odds ratio=0.774, p=0.745). There was no
191 difference in the overall survival of queen and worker larvae in these setups (survival
192 queen larvae: 68% (17/25); survival worker larvae: 80% (20/25); Fisher's exact test,
193 odds ratio=0.538, p=0.52). Workers also reared second instar larvae of both castes
194 to pupation with similar success when they were simultaneously presented with queen
195 and worker larvae in the presence of a mated queen (larvae reared to pupation:
196 queens: 38% (19/50); workers: 29% (14/48); Fisher's exact test, odds ratio=1.482,
197 p=0.398). Finally, when workers reared groups of first and second instar larvae of
198 unknown caste alone or in the presence of a fertile queen, they reared similar
199 proportions of new queens (L1 larvae - queen present: 42.5% queens (17/40); queen
200 absent: 32.6% queens (16/49); Fisher's exact test, odds ratio=1.517, p=0.382; L2
201 larvae - queen present: 13.7% queens (10/73); queen absent: 12.7% queens (7/55);
202 Fisher's exact test, odds ratio=1.089, p=1). Together, these results indicate that *C.*
203 *obscurior* workers are indifferent to the caste of developing individuals.

204 Conclusion

205 Previous studies in *C. obscurior* found that queens control egg sex ratios, and that
206 workers do not manipulate secondary sex ratios of brood [27,28]. In the present study
207 we show that female caste in this species is determined in the egg stage, indicating
208 that caste ratios are also under maternal control. Developing queens and workers
209 differed in size and weight in late larval stages, but not in cuticular odors, hair
210 morphology or physiology. Workers indiscriminately reared eggs and larvae of both
211 castes in different social contexts, including when a fertile queen was present. *C.*
212 *obscurior* queens thus appear to have control over sex and caste allocation, two
213 colony-level traits that often elicit conflict in social Hymenoptera.

214 Queen control of sex allocation has been demonstrated previously in ants [19,51–53],
215 but complete control over caste allocation has, to the best of our knowledge, not

216 been described. In other ant genera with fully sterile workers such as fire ants
217 (*Solenopsis*), Pharaoh ants (*Monomorium*) and big-headed ants (*Pheidole*), caste may
218 similarly be determined in the egg, allowing queens control over primary caste ratios
219 [13,54]; however, workers continue to play an important role in regulating secondary
220 caste ratios by culling sexual larvae in the presence of fertile queens or their
221 pheromonal equivalent [46,55–61].

222 Why then is *Cardiocondyla* different? Colonies are highly inbred due to the evolution
223 of wingless males with lifelong spermatogenesis, which remain in the nest over the
224 course of their relatively long lives to mate with multiple related queens, including
225 sisters and mothers [62–64]. Inbreeding results in particularly high levels of
226 relatedness, and consequently decreased conflict potential within colonies. *C.*
227 *obscurior* moreover has a relatively low degree of dimorphism between sexuals and
228 workers [48,65] and queens only have six ovarioles, producing about 1-2 eggs per
229 day and an average of 350 eggs over the course of their lives [29]. Accordingly,
230 colonies are small and contain on average one to several queens and ~30 workers
231 [65]). In comparison, *Monomorium*, *Solenopsis* and *Pheidole* queens are much larger
232 than their workers and highly fertile, with ovariole numbers ranging from 24 to over
233 100 [15,66], and they can produce hundreds of eggs per day, and hundreds of
234 thousands of eggs over the course of their lives. High queen fertility may allow these
235 species to adopt a strategy of brood culling to dynamically regulate sex and caste
236 allocation, possibly under constraints imposed by high dimorphism between sexuals
237 and workers such as the inability to rear sexuals when colonies are small, lack
238 development stages involved in nutrition processing, or resources are limited
239 [56,67,68]. In *C. obscurior*, low queen fertility means individuals eggs have high
240 relative value for all colony members, likely preventing the evolution of a similar
241 strategy. However, low dimorphism between sexuals and workers allows both castes
242 to be produced at comparable costs, so that even tiny colony fragments consisting
243 of only a few workers and brood can initiate a new colony. This provides an important

244 advantage in the exposed and often disturbed arboreal habitats where the ant thrives,
245 and may help explain its success as a global tramp species [69]. How the unique
246 combination of high relatedness, worker sterility, limited queen fertility and ecological
247 constraints resulted in the evolution of maternal control of egg caste, and the
248 physiological mechanisms underlying this process, remain to be uncovered.

249 Methods

250 Ants

251 *Cardiocondyla obscurior* and several other *Cardiocondyla* species are cosmotropical
252 tramp ants [70]. This myrmicine genus has evolved wingless fighter males with lifelong
253 spermatogenesis, which remain in the nest and mate with newly-hatched nestmate
254 queens [62]. *Cardiocondyla obscurior* has the smallest known ant genome (~193MB,
255 [65,71]). Adult queens and workers differ in size and morphology and workers lack
256 ovaries [48]. Larvae develop via three instars, which can be distinguished by body
257 shape and the degree of melanization of the mandibles [72]. All ants used for this
258 study were from the Old World lineage [71,73], maintained in the lab since 2010.
259 Stock and experimental colonies were kept in plaster-bottom nests with nest inserts
260 made of acrylic glass covered by dark foil in a climate chamber under a 12h/12h and
261 22°C/26°C night/day cycle at 70% humidity. All colonies were provided with water
262 and fed three times a week with honey, pieces of cockroaches and fruit flies.

263 Crystalline deposit patterns in queen and worker larvae of *Cardiocondyla*

264 Localization of crystalline deposits was characterized in eggs, first instar (L1), second
265 instar (L2) and third instar (L3) larvae as queen-like or worker-like by visually inspecting
266 brood from stock colonies under a stereomicroscope. For better detection of the
267 patterns, eggs and L1 larvae were submerged in a dissection dish containing 0.3%
268 PBT, after which they were mounted on a microscope slide and sealed with nail polish.
269 From each developmental stage, representative individuals with queen-like and
270 worker-like patterns were selected and photographed using a stereomicroscope

271 connected to a camera (Keyence VHX 500FD, Neu-Isenburg, Germany). Crystalline
272 deposit patterns were characterized in the same manner in L3 larvae from eight
273 additional *Cardiocondyla* species (*C. "argyrotricha"*, *C. elegans*, *C. emeryi*, *C.*
274 *minutior*, *C. nuda*, *C. thoracica*, *C. venustula*, *C. wroughtonii*) spanning the phylogeny
275 [33]. We further examined larvae of six species from four subfamilies available as live
276 colonies and accessed photos of 36 species from seven subfamilies from Alex Wild's
277 library (https://www.alexanderwild.com/Ants/Natural-History/Metamorphosis-Ant-
278 Brood/), for a total of 51 examined species (Table S2).

279 In *C. obscurior*, the development of all stages was tracked to confirm that crystalline
280 deposit patterns are associated with caste (Table 1). Eggs and L1 larvae were
281 transferred to filter paper to remove excess buffer before being moved in groups of
282 10 to experimental colonies containing 10 workers each (eggs: queen-like=100,
283 worker-like=100, L1 larvae: queen-like=100, worker-like =100). L2 and L3 larvae were
284 transferred in groups of 10-15 to experimental colonies containing 10-12 workers (L2
285 larvae: queen-like=170, worker-like=175; L3 larvae: queen-like=50, worker-like=40).

286 We also tracked the development of L3 larvae from some of the other *Cardiocondyla*
287 species (*C. minutior*, *C. nuda*, *C. thoracica*, *C. venustula*, *C. wroughtonii*) (Table S1).
288 Experimental colonies were monitored three times per week and, when necessary,
289 workers were added from the corresponding stock colonies to standardize worker
290 number. Developing individuals were monitored until pupation, at which time they
291 were counted and classified according to female caste, until no more brood
292 remained. From these data, rates of survival until pupation and caste ratios were
293 calculated. For *C. obscurior*, caste-specific survival and accuracy of caste prediction
294 were tested with Fisher's exact tests (function fisher.test in R 4.0.3 [74]).

295 **Larval histology**

296 *C. obscurior* L3 larvae were collected from stock colonies and sorted according to
297 crystalline deposit patterns. Sorted larvae were transferred into a fixation solution

298 consisting of 25% glutaraldehyde (GAH) in cacodylate buffer [(50 mM cacodylic acid,
299 pH 7.3) containing 150 mM Sucrose] (GAH : cacodylate buffer = 1 : 12,5) and kept
300 overnight at 11 °C. Samples were then rinsed in cacodylate buffer on ice, and fixated
301 in 4% osmium tetroxide in cacodylate buffer. After fixation, larvae were washed in
302 cacodylate buffer, dehydrated in a graded ethanol series and embedded in Epon.
303 Transversal semithin sections of 1 µm were cut and stained with methylene blue and
304 Azur II (Richardson's stain). Semi-thin sections were scanned with a Zeiss Primo Star
305 microscope and imaged with a Moticam 580 digital microscope camera.

306 Phenotypes of *C. obscurior* queen and worker-destined larvae

307 Size

308 Length and width of eggs with queen-like and worker-like crystalline deposit patterns
309 were assessed (n=39 queen-like eggs from 7 stock colonies; n=34 worker-like eggs
310 from 7 stock colonies). Each egg was photographed through a stereomicroscope
311 connected to a camera at 200x magnification and its length and width measured
312 using the system software (Keyence VHX 500FD, Neu-Isenburg, Germany). Caste-
313 specific egg lengths and widths were compared using linear mixed models with
314 length or width as response variables and larval caste as explanatory variable. To
315 account for between-colony variation, the stock colonies from which eggs originated
316 were implemented as random variables. Model residuals were checked for normality
317 using Shapiro-Wilks tests (function shapiro.test) and residual tests implemented in the
318 DHarma package (function testResiduals [75]) in R 4.0.3 [74]. Size differences between
319 castes were assessed using a Wald $\mathbf{X}^2$ test (function Anova, package car [76]).

320 To assess the size of queens and workers over the course of larval development, head
321 capsule width and body length were measured (L1 larvae: n=35 queens, n=32
322 workers; L2 larvae: n=30 queens, n=30 workers; L3 larvae: n=30 queens, n=30
323 workers). Individuals were collected from stock colonies and sorted by instar and
324 caste, and then anaesthetized with CO_2 for four hours. Larvae were placed on their

backs and photographed using a JEOL scanning electron microscope (L1 larvae) or a stereomicroscope connected to a camera (L2 & L3 larvae). Measurements were taken using ImageJ [77] after setting the appropriate scales. In each instar, head capsule width and body length of queen and worker larvae were compared using linear regressions with width or length as response variables and larval caste as explanatory variable. Model residuals were checked for normality using Shapiro-Wilks tests (function shapiro.test) and residual tests (function testResiduals [75]) in R 4.0.3 [74]. Size differences between castes were assessed using a Wald $\mathbf{X}^2$ test (function Anova, package car [76]).

Weight

Five pools of five larvae each per instar (L2, L3) and caste (queen, worker) were collected from stock colonies and weighed on a Sartorius SC2 fine scale (Sartorius, Göttingen, Germany). Weight differences were analysed within each instar with a linear regression using weight per individual larva in mg as response variable and caste as explanatory variable. Model residuals were checked for normality using the Shapiro-Wilks test (function shapiro.test) and residual tests (function testResiduals [75]) and differences between castes were assessed using a Wald $\mathbf{X}^2$ test (function Anova, package car [76]) in R 4.0.3 [74].

Hair density and morphology

Hair density and morphology was assessed qualitatively in queen and worker larvae of all three instars (n=3 per caste and instar). Larvae were collected from stock colonies and sorted by instar and caste, and then placed on a self-adhesive sample insert and moved to -20°C for seven minutes. Freshly killed larvae were scanned with a JEOL scanning electron microscope and density and morphology of hairs was compared visually.

Protein content

351 L2 and L3 queen and worker larvae were collected from stock colonies and fixed
352 individually in 15 µl of 1x PBS solution and frozen at -20°C until further analysis (n=30
353 per instar and caste). For analysis the samples were allowed to thaw on ice and were
354 then homogenized using pestles. Protein concentration in the samples was measured
355 using the Bicinchoninic Acid Protein Assay Kit (Sigma Aldrich, St. Louis, MO, USA)
356 according to the manufacturer's protocol with the following modifications: half of the
357 volume for the microplate assay was used, resulting in 100 µl of working reagent and
358 12.5 µl of sample consisting of 3 µl of homogenized ant sample and 9.5 µl of 1x PBS.
359 All samples were run in two technical replicates at an absorbance of 526 nm using a
360 SpectraMax iD3 microplate reader (Molecular Devices, San Diego, CA, USA). The
361 average of the two technical replicates was used for further calculations. Protein
362 concentration was calculated using a standard curve created with BSA (bovine serum
363 albumin). Within each instar, protein content of queen and worker larvae was
364 compared using a linear regression with protein content in µg/ml as response variable
365 and larval caste as explanatory variable. Model residuals were checked for normality
366 using a Shapiro-Wilks test and residual tests implemented in the DHARMa package.
367 Differences between castes and instars were assessed with a Wald $\mathbf{X}^2$ test. A Levene's
368 test was used to compare the variances of protein content between castes; this was
369 done separately for each instar.

370 Cuticular odors

371 Cuticular hydrocarbon (CHC) profiles of pools of L3 queen-destined larvae (20
372 individuals each, n=6), L3 worker-destined larvae (20 individuals each, n=8), adult
373 queens and adult workers (10 individuals each, n=9 for both groups) were analyzed
374 using gas chromatography coupled with mass-spectrometry (GC-MS). For each
375 sample, individuals from at least five stock colonies were pooled to account for
376 colony-level differences. To obtain samples free of contamination from nest material,
377 individuals used in analyses were collected from stock colonies, separated by caste

378 and kept in freshly-plastered experimental nests for ~24h prior to sampling. All
379 samples were frozen in glass screw top vials and stored at -20°C until extraction.

380 Samples were extracted in 100 µl HPLC grade hexane for 1 minute. Hexane extracts
381 were then fractionated using silica columns (Chromabond SiOH, 1 mL/100mg).
382 Columns were rinsed consecutively with 1 ml of hexane, dichloromethane, methanol
383 and again dichloromethane, before being conditioned with 2 ml of hexane and
384 adding the sample. The non-polar fraction containing hydrocarbons was eluted with
385 1 ml of hexane, and subsequently evaporated under a stream of nitrogen. After being
386 re-dissolved in 100 µl of hexane, 1 µl per sample was subjected to GCMS (Shimadzu
387 GCMS-QP2010 Plus) on a SGE BPX-5 column (30m × 0.25µm × 0.25mm). Helium gas
388 (1 mL/min) served as carrier gas, and injection was splitless. The temperature program
389 started at 70 °C isothermal for 1 min, after which the temperature was raised to 200
390 °C at 30 °C/min and then from 200 to 320 °C at 5 °C/min, where it was held for 5 min.

391 Compound determination of peaks was based on their characteristic mass spectra
392 and matching of retention indices (for methyl-branched alkanes: [78]. For peaks of a
393 concentration too low to yield an unambiguous mass spectrum, compounds were
394 considered to be identical to those present in other samples if their retention indices
395 matched. Compounds that could not be identified were labeled with letters, while
396 double-bond positions of unsaturated hydrocarbons were not further elucidated and
397 are indicated with "x" (Table 2). Only peaks with retention times over 15 minutes
398 (docosane and longer chain lengths) were used for quantitative analyses. Relative
399 peak areas were calculated by dividing individual peak areas by the summed area of
400 all hydrocarbon peaks. Values were then square-root transformed, centered and
401 scaled prior to principal component analysis (PCA, function prcomp in R).
402 Differentiation between groups was tested on square-root transformed values with
403 analysis of similarities (ANOSIM, function anosim in R); tests were run separately for
404 comparisons between developmental stages (adults vs. larvae) and castes within each
405 stage (adult queens vs. adult workers, queen larvae vs. worker larvae).

406 Worker behavior toward queen and worker larvae in different in social contexts

407 The behavior of adults toward queen and worker larvae was investigated in five
408 scenarios.

409 First, we investigated whether isolated worker ants are differentially attracted to L3
410 queen and worker larvae. This was done in round observation chambers (diameter 20
411 mm), which were visually divided into two equally sized halves. In the center of each
412 half, a clean piece of plastic foil with either a L3 queen or worker larva from the same
413 stock colony was placed, so that each chamber contained one larva of each caste.
414 Larvae were glued to the plastic foil with a small drop of sucrose solution to prevent
415 workers from moving larvae around during the observation period. To avoid potential
416 side bias, queen and worker larvae were randomly placed in the left and right halves
417 of the chambers. Once larvae were placed in the chamber, one worker from the same
418 stock colony was added and a transparent microscope slide placed on top to prevent
419 workers from escaping. Chambers were observed for 12 minutes and the number and
420 duration of contacts, i.e., instances of bodily contact between the adult worker and
421 either queen or worker larva, were scored using JWatcher software [79] (n=44
422 chambers observed in total). The number of contacts was analysed with a linear
423 regression using larval caste as the explanatory variable. Model residuals were
424 checked for normality using the Shapiro-Wilks test and residual tests implemented in
425 the DHARMa package. The duration of contacts did not follow a normal distribution
426 and was analysed with a Mann-Whitney-U-test (function wilcox.test) in R.

427 Second, we tested whether worker ants retrieve L3 queen and worker larvae at
428 different rates. From each of five stock colonies, one experimental colony was set up
429 containing 12 workers and one mated queen. Colonies were allowed to settle for 48h.
430 One L3 worker larva and one L3 queen larva from the original stock colonies were
431 then added to the corresponding experimental colonies on a clean piece of filter
432 paper. Experimental colonies were observed until the first larva was retrieved into the

433 nest and the caste of the retrieved larva was noted. This procedure was repeated at
434 least 11 times in each experimental colony, until a total of 67 retrieval events were
435 documented. Retrieval rates of the two castes were compared using a $\mathbf{X}^2$ Test.

436 Third, we investigated whether workers display preferential treatment towards larvae
437 of either caste under food limitation. Five experimental colonies were set up, each
438 containing one mated queen, five workers, and five L2 larvae of each caste. Colonies
439 were then starved of sucrose for three days. On day three, colonies were presented
440 with 0.25 µl of a 1.5 M sucrose solution containing 0.08 g l^{-1} Rhodamine B (Czaczkes
441 et al., 2019). After two hours, all larvae were counted and examined for the presence
442 of Rhodamine B in the digestive tract under a green laser light (555 nm) using a Zeiss
443 Axioplan-2 fluorescence microscope. Larvae positive for Rhodamine B were
444 considered "fed", while the absence of fluorescence in larvae was scored as "unfed".
445 The number of fed and unfed larvae of each caste, as well as the overall survival of
446 queen and worker larvae, was compared using Fisher's exact tests (function fisher.test
447 in R).

448 Fourth, we assessed whether workers changed their rearing behavior in the presence
449 of a mated queen when presented with queen and worker larvae simultaneously. Five
450 colonies were set up, each with 10 second instar queen and 10 second instar worker
451 larvae, as well as 20 workers and one mated queen from the same stock colony.
452 Colonies were fed as described above and all pupae counted and their sex and caste
453 noted. Caste-specific survival of larvae was compared using Fisher's exact test
454 (function fisher.test in R).

455 Fifth, we tested whether the presence of a mated queen influenced secondary caste
456 ratios in brood of unknown caste. First and second instar larvae of unknown caste
457 were collected from stock colonies and reared in experimental colonies with and
458 without queens. For each instar separately, colonies were set up containing 15 larvae
459 and 15 workers, as well as one mated queen (L1 n=10, L2 n=8) or no queen (L1 n=13,

460 L2 n=9). In each experimental colony, all individuals came from the same stock
461 colony. Colonies were fed as described above and all pupae counted and their sex
462 and caste noted. The proportion of queens produced in colonies with and without a
463 mated queen was compared using Fisher's exact test (function fisher.test in R).

464 **Acknowledgements**

465 We thank B. Lautenschläger and M. Schiwek for help with histology, E. Strohm for
466 help identifying the crystalline nature of the deposits, and A. Koch, K. Pogorelski, A.
467 Reisinger and C. Winkler for help with experiments. JO dedicates this paper to Margit
468 Oettler, for nature and nurture. This study was funded by DFG grants (DFG OE 549/3-
469 1, OE 549/3-2, OE 549/3-3).

470 **References**

471 1. Maynard Smith J, Szathmary E. 1995 *The major transitions in evolution*.
472 Oxford, UK: Oxford University Press.

473 2. Boomsma JJ, Gawne R. 2018 Superorganismality and caste differentiation as
474 points of no return: how the major evolutionary transitions were lost in
475 translation. *Biol. Rev.* **93**, 28–54. (doi:10.1111/brv.12330)

476 3. Bourke AFG. 2011 *Principles of social evolution*. Oxford, UK: Oxford
477 University Press.

478 4. Bourke AFG, Franks NR. 1995 *Social Evolution in Ants*. Princeton, NJ:
479 Princeton University Press.

480 5. Keller L. 1995 Social life: the paradox of multiple-queen colonies. *Trends Ecol.*
481 *Evol.* **10**, 355–360.

482 6. Ratnieks FLW, Reeve HK. 1992 Conflict in single-queen Hymenopteran
483 societies: the structure of conflict and processes that reduce conflict in
484 advanced eusocial species. *J. Theor. Biol.* **158**, 33–65.

485 7. Sundström L, Boomsma JJ. 2001 Conflicts and alliances in insect families.

486 *Heredity (Edinb).* **86**, 515–521.

487 8. Heinze J. 2004 Reproductive Conflict in Insect Societies. *Adv. Study Behav.*
488 **34**, 1–57. (doi:10.1016/S0065-3454(04)34001-5)

489 9. Beekman M, Komdeur J, Ratnieks FLW. 2003 Reproductive conflicts in social
490 animals: who has power? *Trends Ecol. Evol.* **18**, 277–282. (doi:10.1016/S0169-
491 5347(03)00068-5)

492 10. Beekman M, Ratnieks FLW. 2003 Power over reproduction in social
493 Hymenoptera. *Philos. Trans. R. Soc. B Biol. Sci.* **358**, 1741–1753.
494 (doi:10.1098/rstb.2002.1262)

495 11. Ratnieks FLW, Foster KR, Wenseleers T. 2006 Conflict resolution in insect
496 societies. *Annu. Rev. Entomol.* **51**, 581–608.
497 (doi:10.1146/annurev.ento.51.110104.151003)

498 12. Khila A, Abouheif E. 2008 Reproductive constraint is a developmental
499 mechanism that maintains social harmony in advanced ant societies. *Proc.*
500 *Natl. Acad. Sci.* **105**, 17884–17889. (doi:10.1073/pnas.0807351105)

501 13. Khila A, Abouheif E. 2010 Evaluating the role of reproductive constraints in
502 ant social evolution. *Philos. Trans. R. Soc. B Biol. Sci.* **365**, 617–630.
503 (doi:10.1098/rstb.2009.0257)

504 14. Ronai I, Vergoz V, Oldroyd BP. 2016 The Mechanistic, Genetic, and
505 Evolutionary Basis of Worker Sterility in the Social Hymenoptera. In *Advances*
506 *in the Study of Behavior*, pp. 251–317. Elsevier Ltd.
507 (doi:10.1016/bs.asb.2016.03.002)

508 15. Gotoh A, Billen J, Hashim R, Ito F. 2016 Degeneration patterns of the worker
509 spermatheca during morphogenesis in ants (Hymenoptera: Formicidae). *Evol.*
510 *Dev.* **18**, 96–104. (doi:10.1111/ede.12182)

511 16. Hammond RL, Keller L. 2004 Conflict over male parentage in social insects.

512 *PLoS Biol.* **2**, E248. (doi:10.1371/journal.pbio.0020248)

513 17. Wenseleers T, Ratnieks FLW. 2006 Comparative analysis of worker
514 reproduction and policing in eusocial hymenoptera supports relatedness
515 theory. *Am. Nat.* **168**, E163–E179. (doi:10.1086/508619)

516 18. Monnin T. 1999 Dominance hierarchy and reproductive conflicts among
517 subordinates in a monogynous queenless ant. *Behav. Ecol.* **10**, 323–332.
518 (doi:10.1093/beheco/10.3.323)

519 19. Rosset H, Chapuisat M. 2006 Sex allocation conflict in ants: when the queen
520 rules. *Curr. Biol.* **16**, 328–331. (doi:10.1016/j.cub.2005.12.036)

521 20. Helanterä H, Ratnieks FLW. 2009 Sex allocation conflict in insect societies:
522 who wins? *Biol. Lett.* **5**, 700–704. (doi:10.1098/rsbl.2009.0501)

523 21. Bourke AFG, Ratnieks FLW. 1999 Kin conflict over caste determination in
524 social Hymenoptera. *Behav. Ecol. Sociobiol.* **46**, 287–297.
525 (doi:https://doi.org/10.1007/s002650050622)

526 22. Mehdiabadi NJ, Reeve HK, Mueller UG. 2003 Queens versus workers: sex-
527 ratio conflict in eusocial Hymenoptera. *Trends Ecol. Evol.* **18**, 88–93.
528 (doi:10.1016/S0169-5347(02)00056-3)

529 23. Dobata S. 2012 Arms race between selfishness and policing: two-trait
530 quantitative genetic model for caste fate conflict in eusocial Hymenoptera.
531 *Evolution (N. Y).* **66**, 3754–3764. (doi:10.1111/j.1558-5646.2012.01745.x)

532 24. Wenseleers T, Ratnieks FLW. 2004 Tragedy of the commons in *Melipona*
533 bees. *Proc. R. Soc. B Biol. Sci.* **271**, S310–S312. (doi:10.1098/rsbl.2003.0159)

534 25. Schultner E, Oettler J, Helanterä H. 2017 The role of brood in eusocial
535 Hymenoptera. *Q. Rev. Biol.* **92**, 39–78. (doi:doi.org/10.1086/690840)

536 26. Schultner E, Pulliainen U. 2020 Brood recognition and discrimination in ants.
537 *Insectes Soc.* **67**, 11–34. (doi:10.1007/s00040-019-00747-3)

538 27. Cremer S, Heinze J. 2002 Adaptive production of fighter males: queens of the
539 ant *Cardiocondyla* adjust the sex ratio under local mate competition. *Proc. R.*
540 *Soc. B Biol. Sci.* **269**, 417–422. (doi:10.1098/rspb.2001.1892)

541 28. De Menten L, Cremer S, Heinze J, Aron S. 2005 Primary sex ratio adjustment
542 by ant queens in response to local mate competition. *Anim. Behav.* **69**, 1031–
543 1035. (doi:10.1016/j.anbehav.2004.09.005)

544 29. Jaimes-Niño LM, Heinze J, Oettler J. 2022 Late-life fitness gains and
545 reproductive death in Cardiocondyla obscurior ants. *Elife* **11**, e74695.

546 30. Paes de Olivaira T V., Da Cruz Landim C. 2003 Morphology and Function of
547 Insect Fat Body Cells: a Review. *Biociencias* **11**, 195–205.

548 31. Zara FJ, Caetano FH. 2004 Ultramorphology and histochemistry of fat body
549 cells from last Instar larval of the *Pachycondyla (=Neoponera) villosa*
550 (Fabricius) (Formicidae: Ponerinae). *Brazilian J. Biol.* **64**, 725–735.
551 (doi:10.1590/s1519-69842004000400022)

552 32. Elliott KL, Stay B. 2007 Juvenile hormone synthesis as related to egg
553 development in neotenic reproductives of the termite *Reticulitermes flavipes*,
554 with observations on urates in the fat body. *Gen. Comp. Endocrinol.* **152**,
555 102–110. (doi:10.1016/j.ygcen.2007.03.003)

556 33. Oettler J, Suefuji M, Heinze J. 2010 The evolution of alternative reproductive
557 tactics in male *Cardiocondyla* ants. *Evolution (N. Y).* **64**, 3310–3317.
558 (doi:10.1111/j.1558-5646.2010.01090.x)

559 34. Klein A *et al.* 2016 A novel intracellular mutualistic bacterium in the invasive
560 ant *Cardiocondyla obscurior. ISME J.* **10**, 376–388.
561 (doi:10.1038/ismej.2015.119)

562 35. Jackson R *et al.* 2022 Convergent evolution of a labile nutritional symbiosis in
563 ants. *ISME J.* **16**, 2114–2122. (doi:10.1038/s41396-022-01256-1)

36. Ün Ç, Hacker J, Heinze J, Oettler J, Schultner E. 2021 Endosymbionts mediate the effects of antibiotic exposure in the tramp ant *Cardiocondyla obscurior*. *Ecol. Entomol.* , 13099. (doi:10.1111/een.13099)

37. Wheeler GC, Wheeler J. 1976 Ant Larvae: Review and Synthesis. *Mem. Entomol. Soc. Washingt.* **7**, 1–108.

38. Wheeler GC, Wheeler J. 1986 Ten-year supplement to 'Ant larvae: review and synthesis'. *Proc. Entomol. Soc. Washingt.* **88**, 684–702.

39. Wheeler W. 1918 A study of some ant larvae, with a consideration of the origin and meaning of the social habit among insects. *Proc. Am. Philos. Soc.* **57**, 293–343.

40. Solis DR, Fox EGP, Kato LM, de Jesus CM, Yabuki AT, Campos AEDC, Bueno OC. 2010 Morphological description of the immatures of the ant, *Monomorium floricola*. *J. Insect Sci.* **10**, 15. (doi:10.1673/031.010.1501)

41. Penick CA, Ebie J, Moore D. 2014 A non-destructive method for identifying the sex of ant larvae. *Insectes Soc.* **61**, 51–55. (doi:10.1007/s00040-013-0323-5)

42. Villalta I, Amor F, Cerdá X, Boulay R. 2016 Social coercion of larval development in an ant species. *Sci. Nat.* **103**, 18. (doi:10.1007/s00114-016-1341-8)

43. Penick CA, Liebig J. 2017 A larval 'princess pheromone' identifies future ant queens based on their juvenile hormone content. *Anim. Behav.* **128**, 33–40. (doi:10.1016/j.anbehav.2017.03.029)

44. Brian MV. 1973 Queen recognition by brood-rearing workers of the ant *Myrmica rubra* L. *Anim. Behav.* **21**, 691–698. (doi:10.1016/S0003-3472(73)80093-4)

45. Passera L, Aron S, Bach D. 1995 Elimination of sexual brood in the Argentine

590 ant *Linepithema humile*: queen effect and brood recognition. *Entomol. Exp.*
591 *Appl.* **75**, 203–212. (doi:10.1111/j.1570-7458.1995.tb01928.x)

592 46. Edwards J. 1991 Caste regulation in the pharaoh's ant *Monomorium*
593 *pharaonis*: recognition and cannibalism of sexual brood by workers. *Physiol.*
594 *Entomol.* **16**, 263–271. (doi:doi.org/10.1111/j.1365-3032.1991.tb00565.x)

595 47. Adams RMM, Larsen RS, Stylianidi N, Cheung D, Qiu B, Murray S, Zhang G,
596 Boomsma J. 2021 Hairs distinguish castes and sexes: identifying the early
597 ontogenetic building blocks of a fungus-farming superorganism.
598 *Myrmecological News* **31**, 201–216. (doi:10.25849/myrmecol.news)

599 48. Oettler J, Platschek T, Schmidt C, Rajakumar R, Favé M-J, Khila A, Heinze J,
600 Abouheif E. 2019 Interruption points in the wing gene regulatory network
601 underlying wing polyphenism evolved independently in male and female
602 morphs in *Cardiocondyla* ants. *J. Exp. Zool. Part B Mol. Dev. Evol.* **332**, 7–16.
603 (doi:10.1002/jez.b.22834)

604 49. Holman L, Jørgensen CG, Nielsen J, d'Ettorre P. 2010 Identification of an ant
605 queen pheromone regulating worker sterility. *Proc. R. Soc. B Biol. Sci.* **277**,
606 3793–3800. (doi:10.1098/rspb.2010.0984)

607 50. Endler A, Liebig J, Schmitt T, Parker JE, Jones G, Schreier P, Hölldobler B.
608 2004 Surface hydrocarbons of queen eggs regulate worker reproduction in a
609 social insect. *Proc. Natl. Acad. Sci.* **101**, 2945–2950.
610 (doi:10.1073/pnas.0308447101)

611 51. Passera L, Aron S, Vargo EL, Keller L. 2001 Queen control of sex ratio in fire
612 ants. *Science (80-.).* **293**, 1308–1310. (doi:10.1126/science.1062076)

613 52. De Menten L, Fournier D, Brent CS, Passera L, Vargo EL, Aron S. 2005 Dual
614 mechanism of queen influence over sex ratio in the ant *Pheidole pallidula*.
615 *Behav. Ecol. Sociobiol.* **58**, 527–533. (doi:10.1007/s00265-005-0964-0)

616 53. Aron S, Passera L, Keller L. 1994 Queen-worker conflict over sex ratio: A
617 comparison of primary and secondary sex ratios in the Argentine ant,
618 *Iridomyrmex humilis*. *J. Evol. Biol.* **7**, 403–418. (doi:10.1007/s10958-007-0251-
619 x)

620 54. Passera L, Suzzoni JP. 1979 Le role de la reine de *Pheidole pallidula* (Nyl.)
621 (Hymenoptera, Formicidae) dans la sexualisation du couvain après traitement
622 par l'hormone juvénile. *Insectes Soc.* **26**, 343–353. (doi:10.1007/BF02223553)

623 55. Klobuchar E, Deslippe R. 2002 A queen pheromone induces workers to kill
624 sexual larvae in colonies of the red imported fire ant (*Solenopsis invicta*).
625 *Naturwissenschaften* **89**, 302–304. (doi:10.1007/s00114-002-0331-1)

626 56. Passera L. 1980 La ponte d'œufs préorientés chez la fourmi *Pheidole pallidula*
627 (Nyl.) (Hymenoptera-Formicidae). *Insectes Soc.* **27**, 79–95.
628 (doi:10.1007/BF02224522)

629 57. Vargo EL, Passera L. 1991 Pheromonal and behavioral queen control over the
630 production of gynes in the Argentine ant *Iridomyrmex humilis* (Mayr). *Behav.*
631 *Ecol. Sociobiol.* **28**, 161–169.

632 58. Edwards J. 1987 Caste regulation in the pharaoh's ant *Monomorium*
633 *pharaonis*: the influence of queens on the production of new sexual forms.
634 *Physiol. Entomol.* **12**, 31–39. (doi:doi.org/10.1111/j.1365-
635 3032.1987.tb00721.x)

636 59. Warner MR, Lipponen J, Linksvayer TA. 2018 Pharaoh ant colonies
637 dynamically regulate reproductive allocation based on colony demography.
638 *Behav. Ecol. Sociobiol.* **72**, 31. (doi:10.1007/s00265-017-2430-1)

639 60. Boonen S, Billen J. 2017 Caste regulation in the ant *Monomorium pharaonis*
640 (L.) with emphasis on the role of queens. *Insectes Soc.* **64**, 113–121.
641 (doi:10.1007/s00040-016-0521-z)

642 61. Oliveira RC, Warson J, Sillam-Dussès D, Herrera-Malaver B, Verstrepen K,
643 Millar JG, Wenseleers T. 2020 Identification of a queen pheromone mediating
644 the rearing of adult sexuals in the pharaoh ant *Monomorium pharaonis*. *Biol.*
645 *Lett.* **16**, 20200348. (doi:10.1098/rsbl.2020.0348rsbl20200348)

646 62. Heinze J, Hölldobler B. 1993 Fighting for a harem of queens: physiology of
647 reproduction in *Cardiocondyla* male ants. *Proc. Natl. Acad. Sci.* **90**, 8412–
648 8414.

649 63. Heinze J. 2016 The male has done his work - The male may go. *Curr. Opin.*
650 *Insect Sci.* **16**, 22–27. (doi:10.1016/j.cois.2016.05.005)

651 64. Schmidt CV, Frohschammer S, Schrempf A, Heinze J. 2014 Virgin ant queens
652 mate with their own sons to avoid failure at colony foundation.
653 *Naturwissenschaften* **101**, 69–72. (doi:10.1007/s00114-013-1126-2)

654 65. Schrader L *et al.* 2014 Transposable element islands facilitate adaptation to
655 novel environments in an invasive species. *Nat. Commun.* **5**, 5495.
656 (doi:10.1038/ncomms6495)

657 66. Tschinkel WR. 1987 Relationship between Ovariole Number and
658 Spermathecal Sperm Count in Ant Queens: a New Allometry. *Ann. Entomol.*
659 *Soc. Am.* **80**, 208–211. (doi:10.1093/aesa/80.2.208)

660 67. Warner MR, Kovaka K, Linksvayer TA. 2016 Late-instar ant worker larvae play a
661 prominent role in colony-level caste regulation. *Insectes Soc.* **63**, 1–9.
662 (doi:10.1007/s00040-016-0501-3)

663 68. Tschinkel WR. 1995 Stimulation of fire ant queen fecundity by a highly specific
664 brood stage. *Ann. Entomol. Soc. Am.* **88**, 876–882.

665 69. Heinze J. 2017 Life-history evolution in ants: the case of *Cardiocondyla*. *Proc.*
666 *R. Soc. B Biol. Sci.* **284**, 20161406. (doi:10.1098/rspb.2016.1406)

667 70. Heinze J, Cremer S, Eckl N, Schrempf A. 2006 Stealthy invaders: The biology

668 of *Cardiocondyla* tramp ants. *Insectes Soc.* **53**, 1–7. (doi:10.1007/s00040-005-
669 0847-4)

670 71. Errbii M, Keilwagen J, Hoff KJ, Steffen R, Altmüller J, Oettler J, Schrader L.
671 2021 Transposable elements and introgression introduce genetic variation in
672 the invasive ant *Cardiocondyla obscurior*. *Mol. Ecol.* , mec.16099.
673 (doi:10.1111/mec.16099)

674 72. Schrempf A, Heinze J. 2006 Proximate mechanisms of male morph
675 determination in the ant *Cardiocondyla obscurior*. *Evol. Dev.* **8**, 266–272.
676 (doi:10.1111/j.1525-142X.2006.00097.x)

677 73. Ün Ç, Schultner E, Manzano-Marín A, Flórez L V., Seifert B, Heinze J, Oettler
678 J. 2021 Cytoplasmic incompatibility between Old and New World populations
679 of a tramp ant. *Evolution (N. Y).* **75**, 1775–1791. (doi:10.1111/evo.14261)

680 74. R Team. 2016 R: A Language and Environment for Statistical Computing.

681 75. Hartig F. 2020 DHARMa: Residual Diagnostics for Hierarchical (Multi-Level /
682 Mixed) Regression Models.

683 76. Fox J, Weisberg S. 2019 *An R Companion to Applied Regression*. Third edit.
684 Thousand Oaks, CA: Sage. See
685 https://socialsciences.mcmaster.ca/jfox/Books/Companion/.

686 77. Schneider C, Rasband W, Eliceiri K. 2012 NIH Image to ImageJ: 25 years of
687 image analysis. *Nat. Methods* **9**, 671–675.
688 (doi:https://doi.org/10.1038/nmeth.2089)

689 78. Carlson DA, Bernier UR, Sutton BD. 1998 Elution Patterns from Capillary GC
690 for Methyl-Branched Alkanes. *J. Chem. Ecol.* **24**, 1845–1865.
691 (doi:10.1023/A:1022311701355)

692 79. Blumstein DT, Daniel J. 2007 *Quantifying Behavior the JWatcher way*.
693 Sunderland, MA: Sinauer Associates.

694

695 Figure legends

696 Figure 1: Crystalline deposit patterns distinguish queen- and worker-destined larvae
697 in the ant *Cardiocondyla obscurior*. Stereomicroscope images of queen-destined and
698 worker-destined larval instars show a pair of large, clumped crystalline deposits (cd)
699 in the posterior end of queens, and small, randomly distributed crystalline deposits
700 in workers (lateral and dorsal views).

701 Figure 2: Crystalline deposit patterns in queen- and worker-destined embryos in the
702 ant *Cardiocondyla obscurior*. Queen-destined embryos have crystalline deposits (cd)
703 localized in the last segments of their germ band (gb). Worker-destined embryos do
704 not show any crystalline deposits. The first segments of the embryo are separated
705 into the head and the three gnathal segments: mandibular (md), maxillary (mx) and
706 labial (lb).

707 Figure 3: Histological sections of queen and worker-destined third instar larvae. (A)
708 Queen-destined larvae show paired ovaries (ov) close to the midgut (mid). (B,C)
709 Ovaries in queen-destined larvae are surrounded by large crystalline deposits. (D) In
710 workers ovaries are missing. (E, F) In worker-destined larvae crystalline deposits are
711 distributed randomly and aggregate close to trophocytes (t). top row: 5x
712 magnification, middle row: 20x magnification, bottom row: 40x magnification.

713 Figure 4: Size of *Cardiocondyla obscurior* queen and worker eggs and larvae (A) egg
714 width, (B) egg length, (C) larva head capsule width, (D) larva body length. L1: first
715 instar, L2: second instar, L3: third instar

716 Figure 5: Weight (A) and protein content (B) of queen and worker *Cardiocondyla*
717 *obscurior* larvae. L2: second instar, L3: third instar

718 Figure 6: Principal component analysis of cuticular hydrocarbon profiles of queen and
719 worker larvae and adults in the ant *Cardiocondyla obscurior*

720

Figures

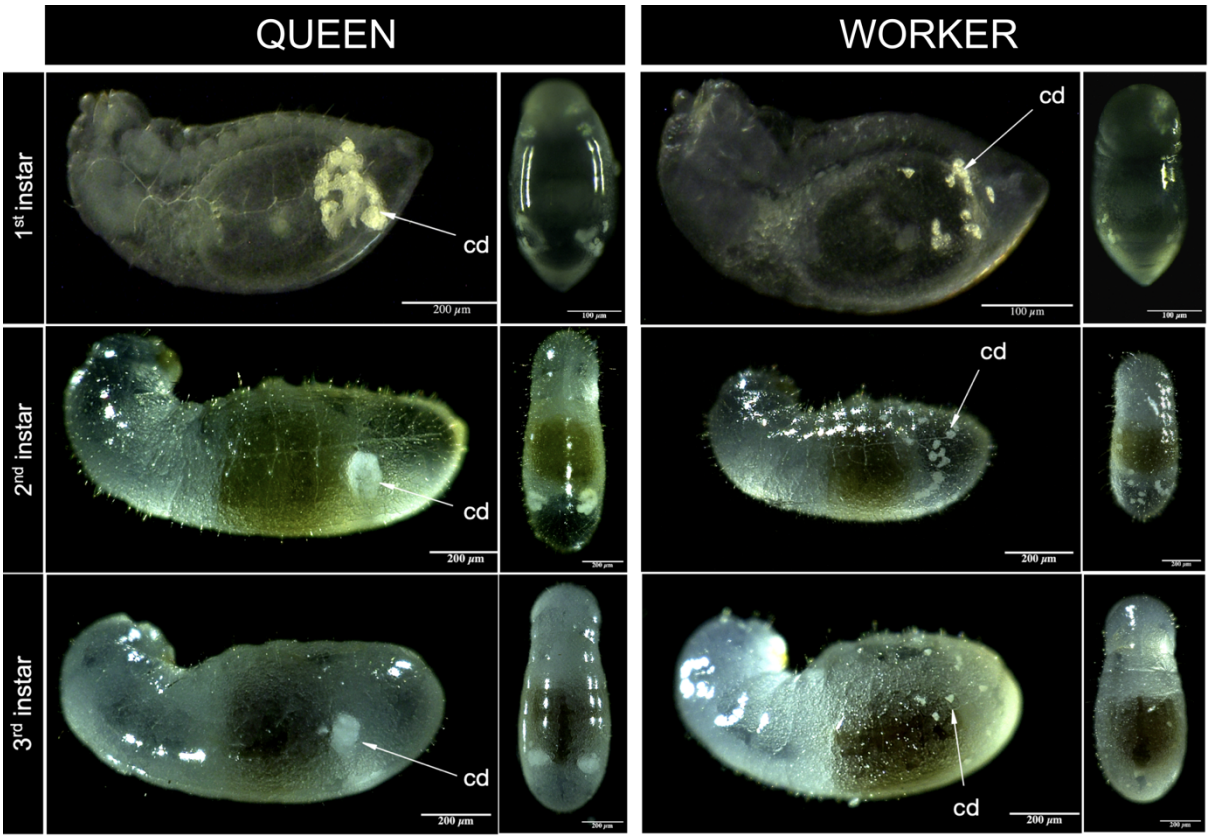


Figure 1: Crystalline deposit patterns distinguish queen- and worker-destined larvae in the ant *Cardiocondyla obscurior*. Stereomicroscope images of queen-destined and worker-destined larval instars show large, paired crystalline deposits (cd) in the posterior end of queens, and small, randomly distributed crystalline deposits in workers (lateral and dorsal views).

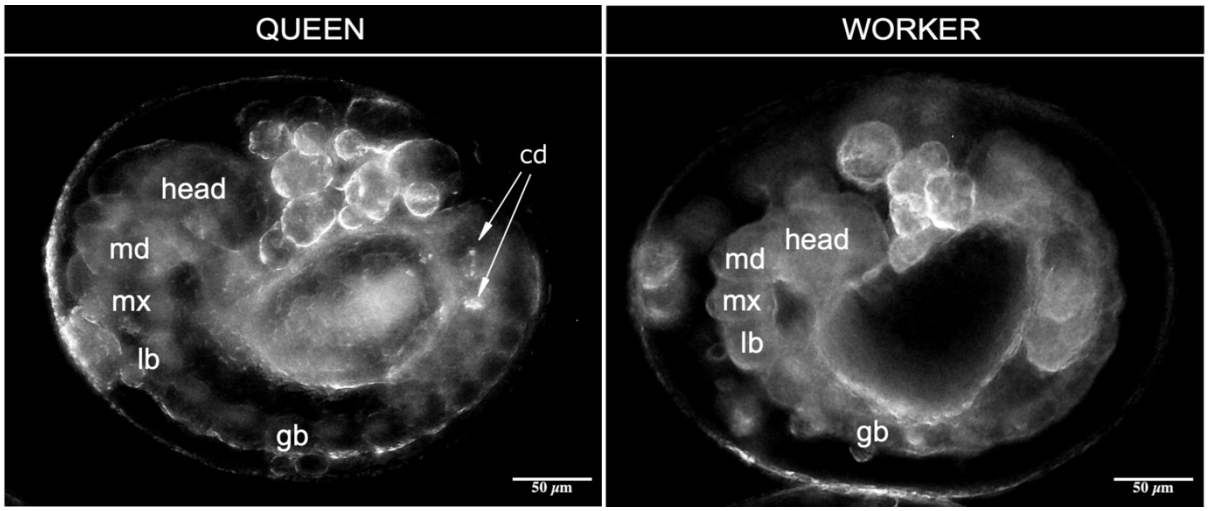


Figure 2: Crystalline deposit patterns in queen- and worker-destined embryos in the ant *Cardiocondyla obscurior*. Queen-destined embryos have crystalline deposits (cd) localized in the last segments of their germ band (gb). Worker-destined embryos do not show any crystalline deposits. The first segments of the embryo are clearly separated into the head and the three gnathal segments: mandibular (md), maxillary (mx) and labial (lb).

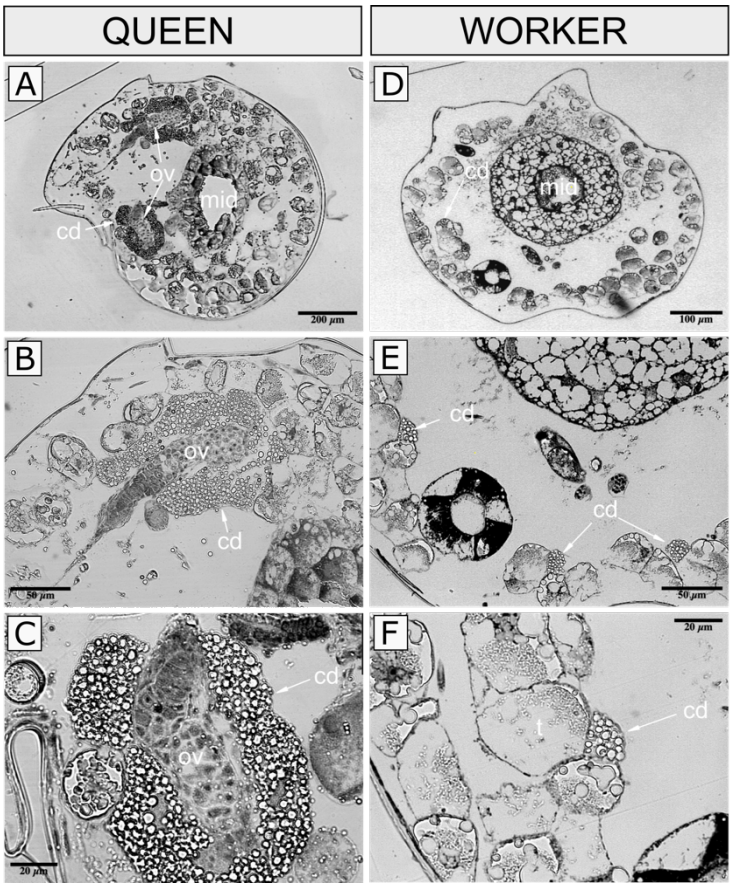


Figure 3: Histological sections of queen and worker-destined third instar larvae. (A) Queen-destined larvae show paired ovaries (ov) close to the midgut (mid). (B,C) Ovaries in queen-destined larvae are surrounded by large crystalline deposits. (D) Transversal section of a worker-destined L3 larva; in workers ovaries are missing. (E, F) In worker-destined larvae crystalline deposits are distributed randomly and aggregate close to trophocytes (t). top row: 5x magnification, middle row: 20x, bottom row: 40x.

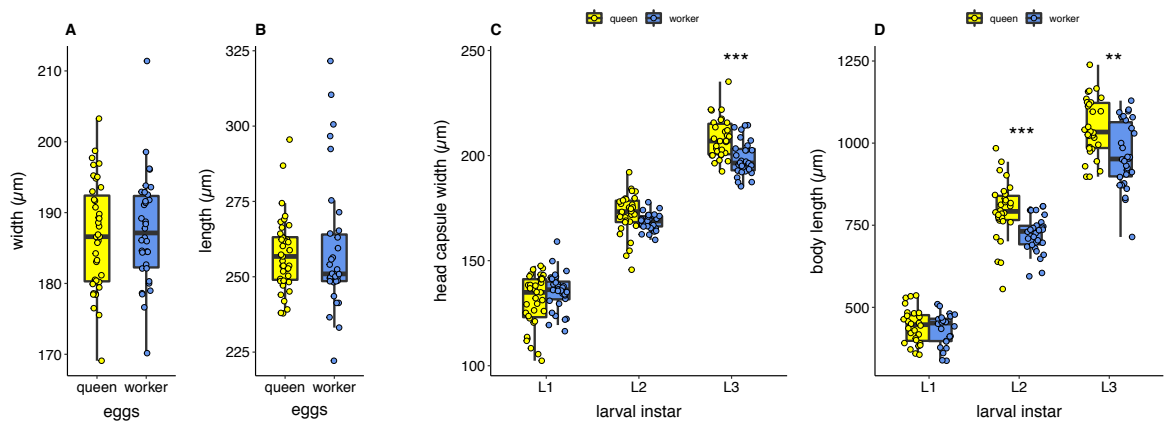


Figure 4: Size of *C. obscurior* queen and worker eggs and larvae (A) egg width, (B) egg length, (C) larva head capsule width, (D) larva body length. L1: first instar, L2: second instar, L3: third instar

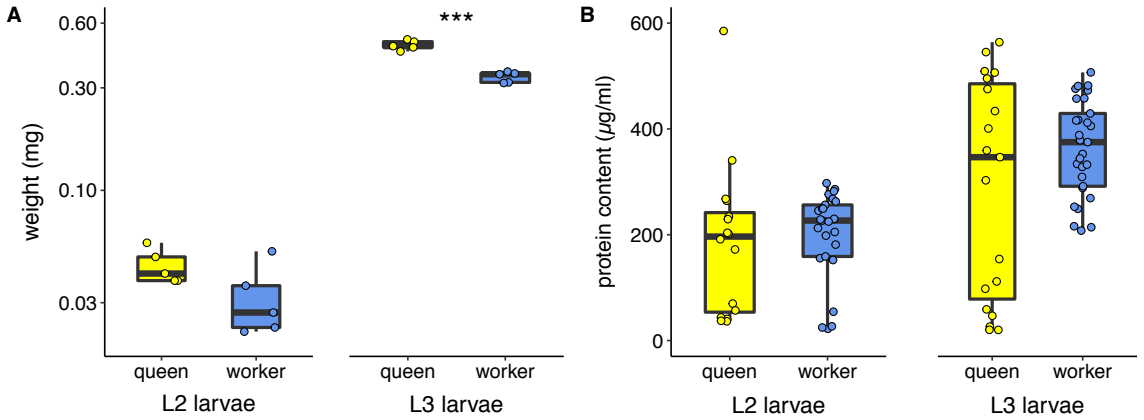


Figure 5: Weight (A) and protein content (B) of queen and worker ant larvae L2: second instar, L3: third instar

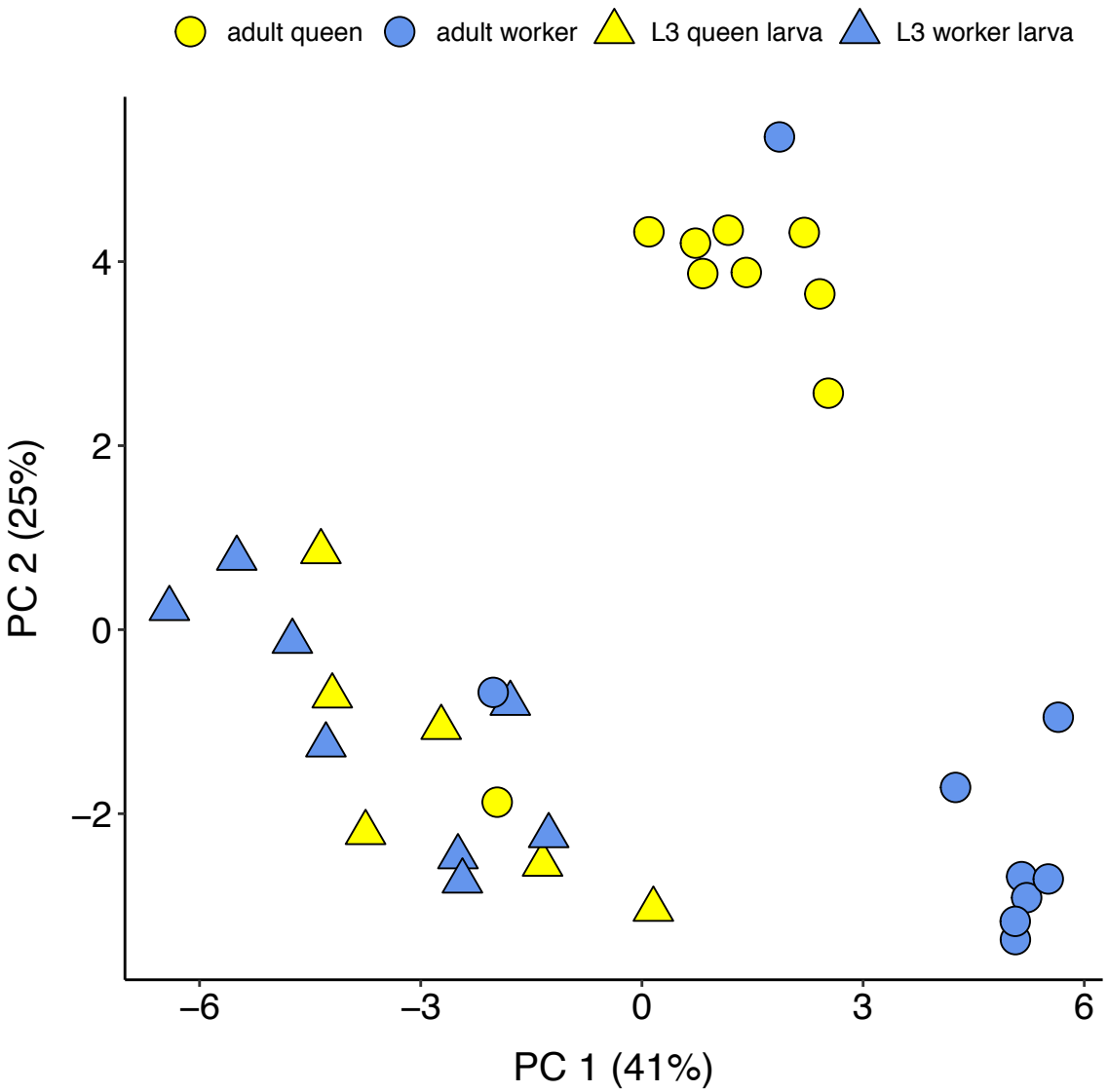


Figure 6: Principal component analysis of cuticular hydrocarbon profiles of queen and worker larvae and adults in the ant *Cardiocondyla obscurior*

Table 1: Localization of crystalline deposits predicts caste in eggs and larvae of the ant *Cardiocondyla obscurior*

Development Stage	Crystalline deposit pattern	n	Proportion of pupae produced	Survival differences between predicted castes		Proportion of correct predictions	Accuracy of caste prediction	
				Odds ratio	p		Odds ratio	p
egg	queen-like	100	56% (56/100)	0.664	0.157	82.1% (46/56)	83.033	**<0.001**
	worker-like	100	45% (45/100)			95.1% (39/41)		
1st instar larva	queen-like	100	47% (47/100)	0.961	1	57.4% (27/47)	28.573	**<0.001**
	worker-like	100	46% (46/100)			95.6% (44/46)		
2nd instar larva	queen-like	170	58.2% (99/170)	0.648	0.052	96.9% (96/99)	2133.229	**<0.001**
	worker-like	175	47.4% (83/175)			98.8% (82/83)		
3rd instar larva	queen-like	50	60% (30/50)	0.497	0.137	100% (30/30)	infinite	**<0.001**
	worker-like	40	42.5% (17/40)			100% (17/17)		

Note that 4 out 45 of eggs predicted to be workers developed into males; these were excluded prior to analysis of caste prediction accuracy. No males developed in any of the other setups.

Table 2: Cuticular hydrocarbon profiles of queen and worker larvae and adults of the ant *Cardiocondyla obscurior*

		mean relative amounts ± standard deviation (%)			
RI	Compound	Queen larvae	Worker larvae	Queens	Workers
2189	C22	3.78 ± 2.66	3.89 ± 2.83	2.11 ± 1.29	6.25 ± 2.49
2300	A[a]	1.2 ± 0.72	0.83 ± 0.57	0.51 ± 0.12	1.49 ± 0.52
2400	C24	2.97 ± 1.71	2.77 ± 1.68	1.63 ± 0.89	3.28 ± 0.64
2499	C25	50.49 ± 7.14	50.37 ± 4.92	26.28 ± 8.93	13.48 ± 6.99
2532	13-Me C25 [b]	tr	tr	0.51 ± 0.16	1.25 ± 0.64
2572	3-Me C25	3.27 ± 1.68	2.61 ± 1.14	4.66 ± 1.22	8.04 ± 1.28
2599	C26	3.13 ± 0.96	3.18 ± 1.19	2.47 ± 0.82	3.5 ± 0.53
2632	B	tr	tr	tr	0.64 ± 0.41
2662	C	tr	tr	tr	tr
2675	x,x-C27:2	1.1 ± 0.42	0.59 ± 0.31	7.28 ± 1.27	13.24 ± 7.64
2699	C27	17.89 ± 4.13	20.8 ± 3.68	25.68 ± 5.05	9.65 ± 6.68
2731	13-Me, 11-Me C27	1.46 ± 0.76	0.94 ± 0.44	3.3 ± 1.39	12.56 ± 5.46
2749	5-Me C27	tr	tr	tr	0.58 ± 0.26
2771	3-Me C27	1.79 ± 0.37	1.61 ± 0.35	2.66 ± 0.5	2.35 ± 0.42
2799	D[a]	1.63 ± 0.74	1.5 ± 0.75	1.89 ± 0.73	2.69 ± 0.8
2831	E	tr	tr	tr	1.06 ± 0.51
2861	4-Me C28	0.61 ± 0.27	0.6 ± 0.44	tr	tr
2880	x-C29:1	tr	tr	tr	tr
2899	C29	3.7 ± 1.33	4.3 ± 1.07	8.73 ± 2.52	4.24 ± 2.58
2929	13-Me, 11-Me C29	1.02 ± 0.53	tr	1.45 ± 0.42	5.39 ± 2.21
2972	3-Me C29	tr	tr	0.8 ± 0.31	tr
2999	C30	1.68 ± 0.81	1.94 ± 0.8	1.61 ± 1.38	4.36 ± 1.77
3006	F	tr	tr	0.59 ± 0.19	tr
3028	G	tr	tr	tr	tr
3060	H	tr	tr	tr	tr
3079	x-C31:1	tr	tr	tr	tr
3098	C31	0.57 ± 0.18	0.54 ± 0.23	1.88 ± 0.76	tr
3125	13-Me C31 [b]	0.91 ± 0.42	tr	2.28 ± 0.71	1.85 ± 0.9
3172	3-Me C31	tr	tr	tr	tr
3196	I	tr	tr	0.66 ± 0.28	0.52 ± 0.25
3297	C33	0.69 ± 0.42	0.77 ± 0.61	0.93 ± 0.52	1.16 ± 0.72
3326	13-Me C33 [b]	tr	tr	tr	0.77 ± 0.42

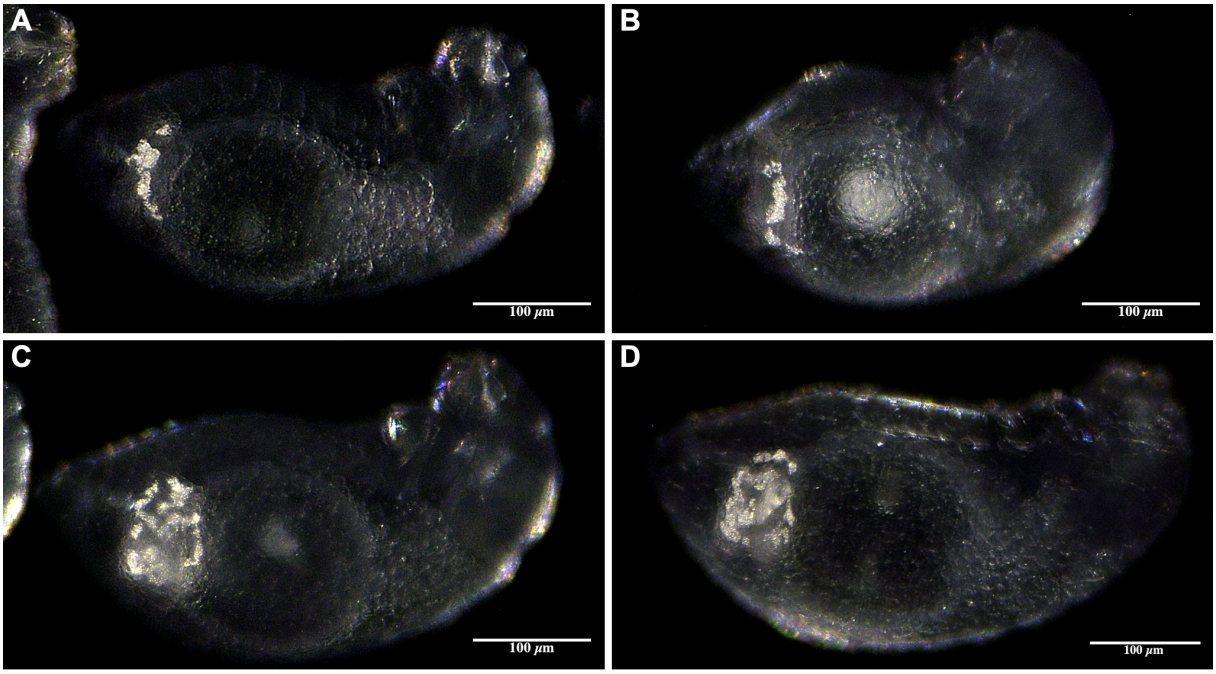
RI - linear retention index

tr – trace amounts (<0.5% of total profile)

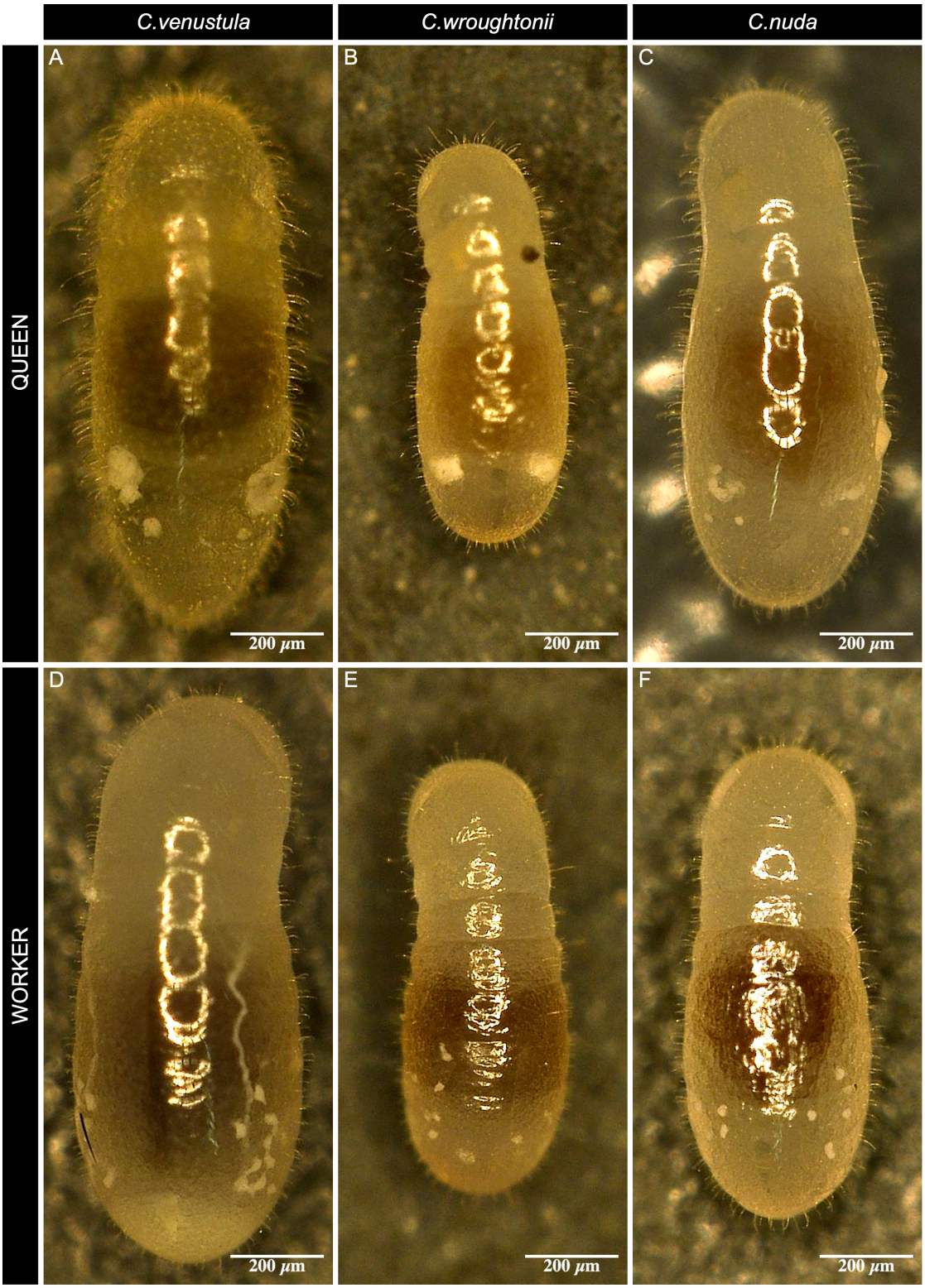
[a] diagnostic ions were not detectable, but RIs indicate: A=C23, D=C28

[b] peaks may include a further monomethyl alkane (11-Me) of the respective chain length at low relative concentration

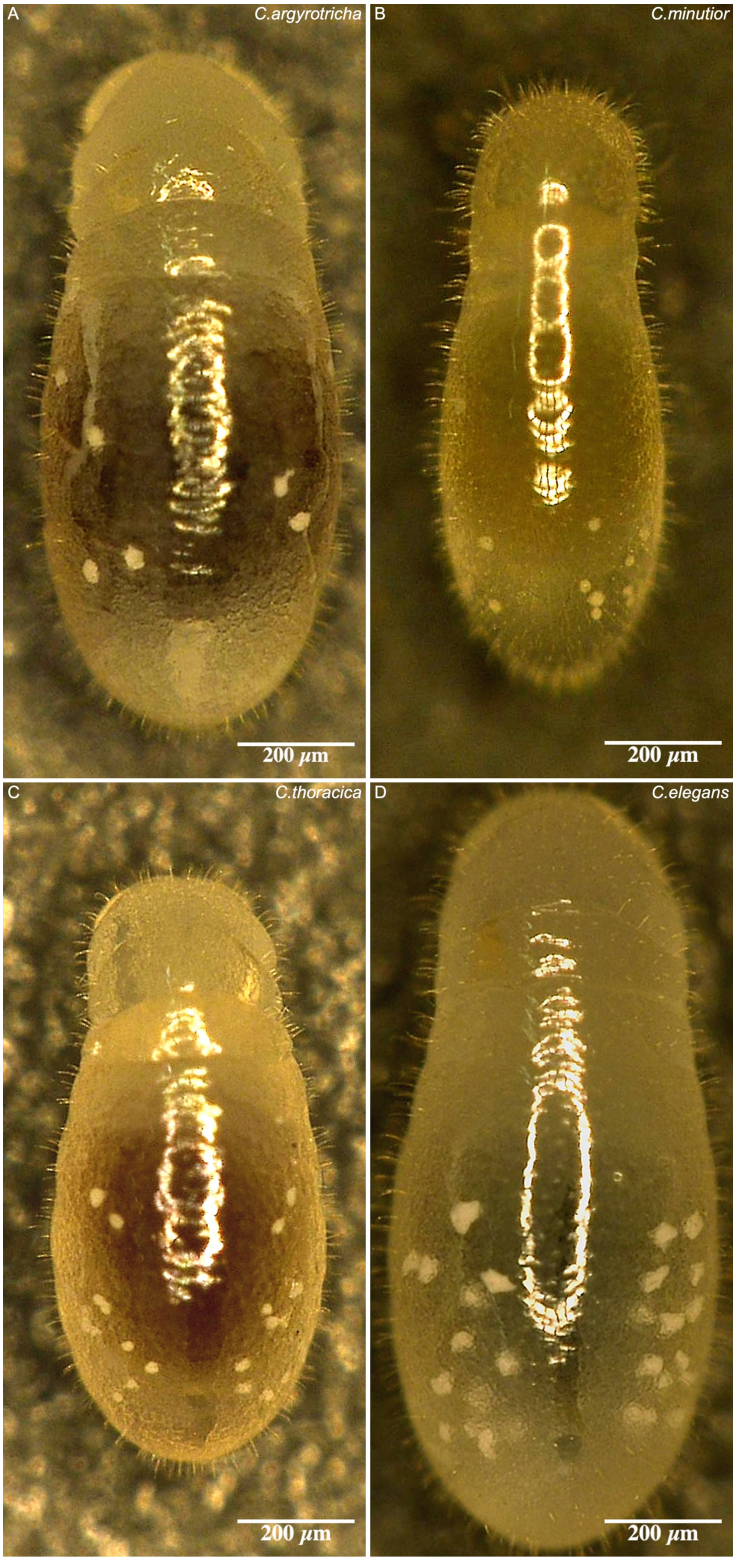
Supplementary Figure S1: Crystalline deposit patterns are variable in first instar queen-destined larvae. (A,B) String of pearl-like deposits. (C,D) Blob-like deposits.



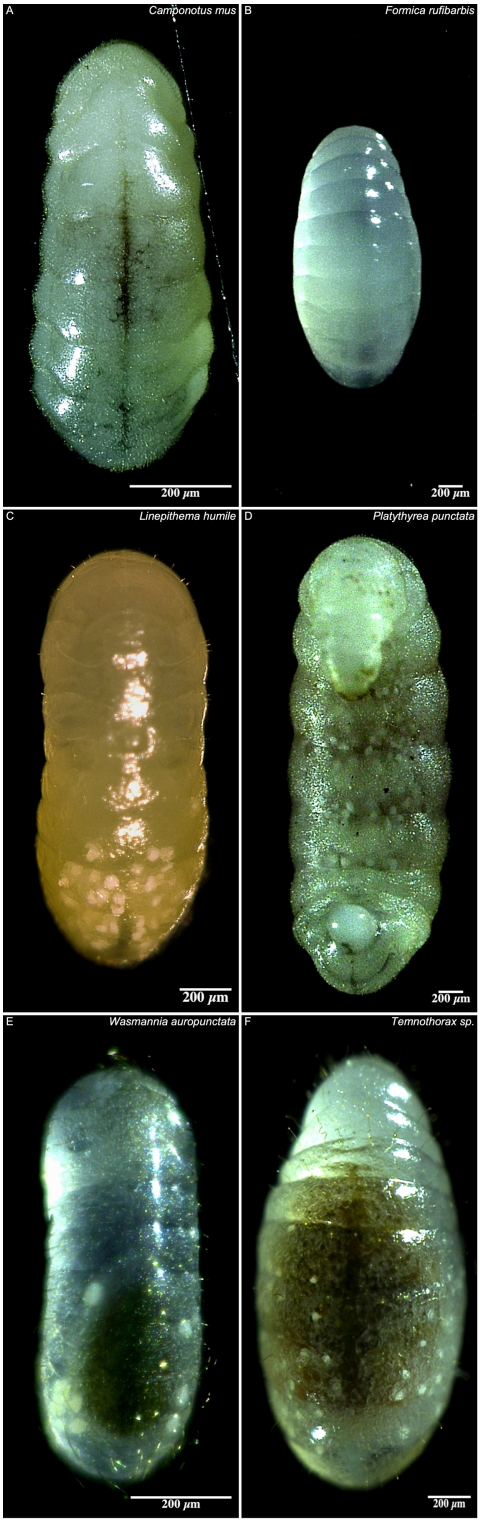
Supplementary Figure S2: Crystalline deposit patterns distinguish queen- and worker-destined larvae in several *Cardiocondyla* species. Stereomicroscope images of queen-destined (A, B, C) and worker-destined (D, E, F) third instar larvae in *C. venustula, C. wroughtonii* and *C. nuda*.



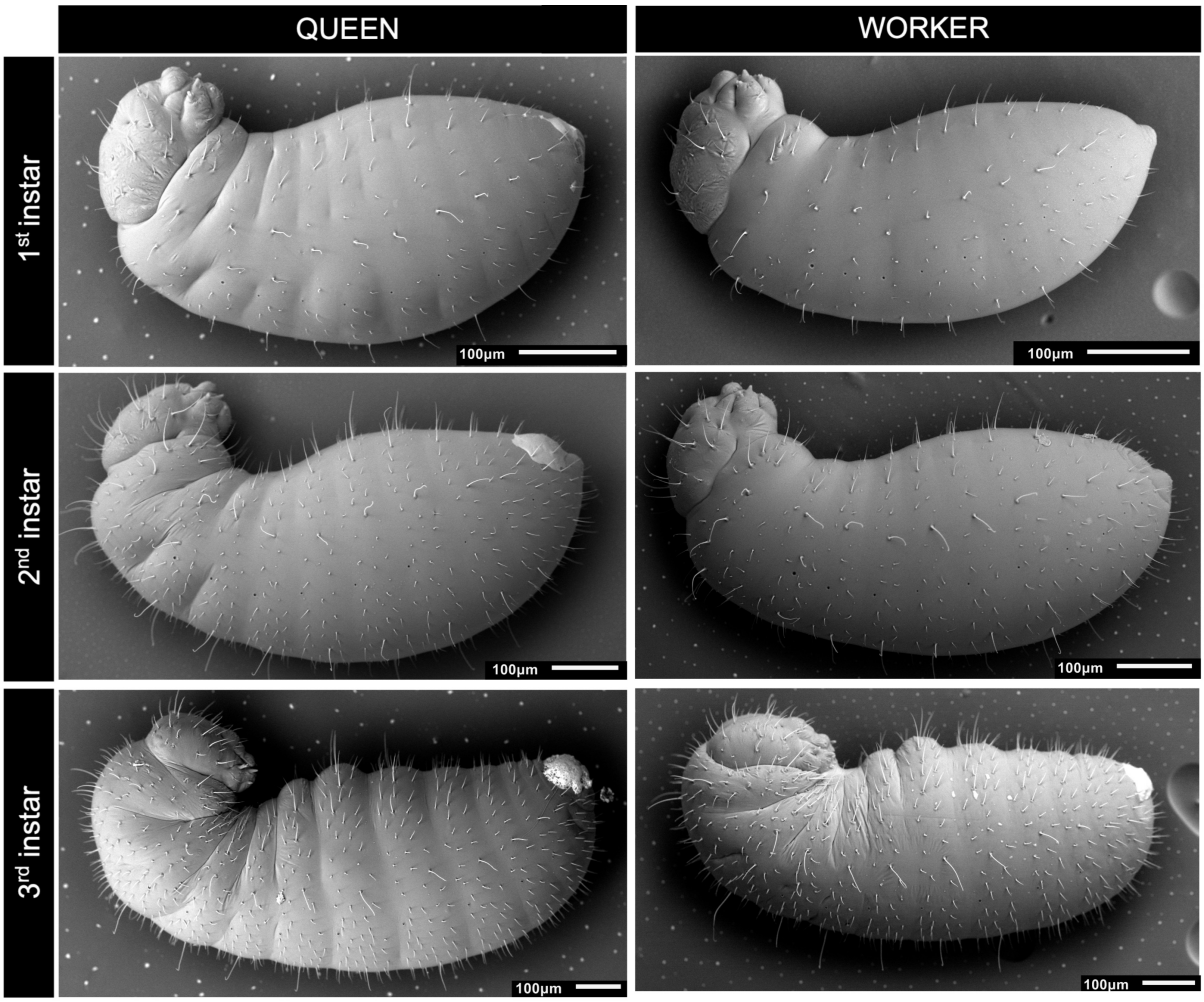
Supplementary Figure S3: Crystalline deposit patterns in *Cardiocondyla* species without caste specific patterns. Stereomicroscope images of third instar larvae in *C. "argyrotricha"*, *C. minutior*, *C. thoracica* and *C. elegans*. No pictures were taken of *C. emeryi*.



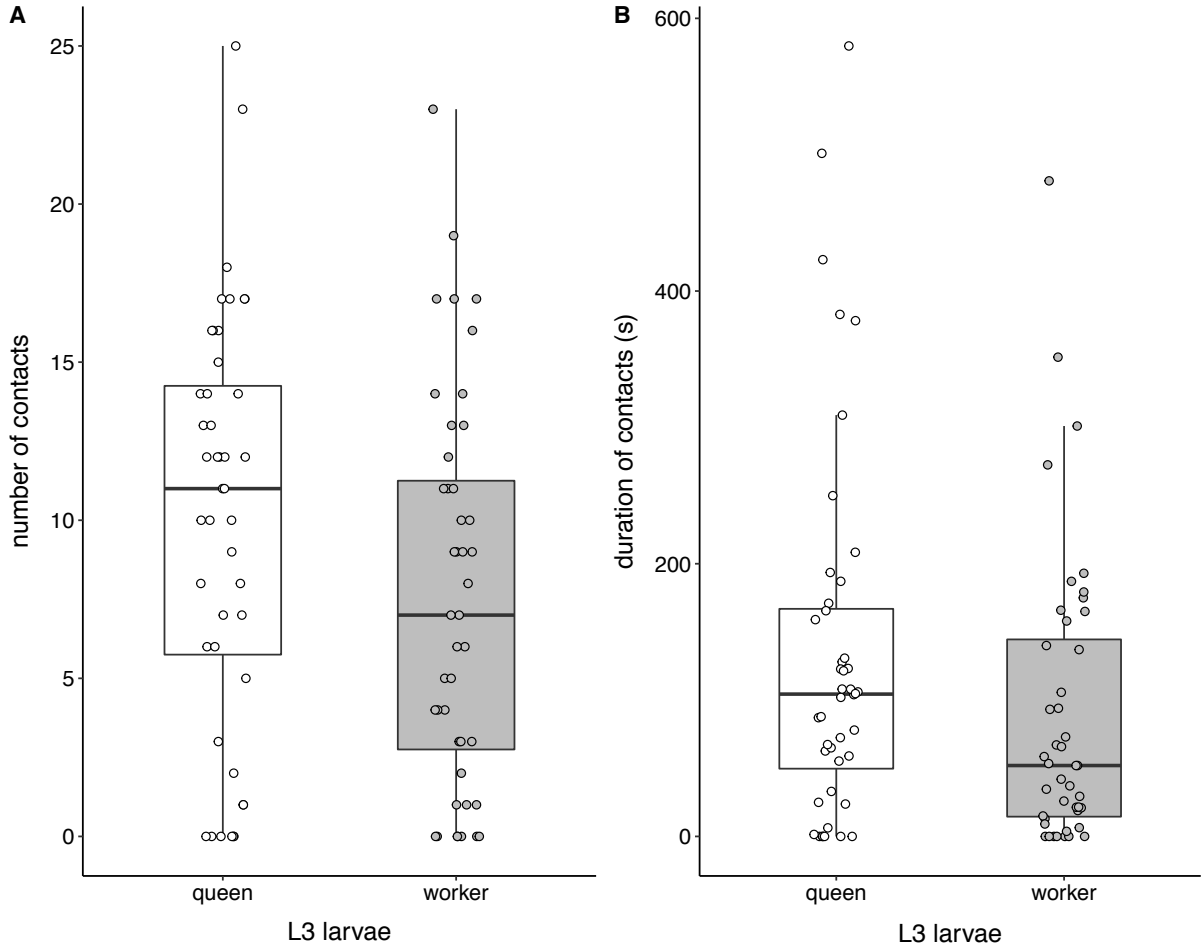
Supplementary Figure S4: Crystalline deposit patterns in seven species from four ant subfamilies available as live colonies in the lab. Stereomicroscope images of larvae in *Camponotus mus* (Formicinae), *Formica rufibarbis* (Formicinae), *Linepithema humile* (Dolichoderinae), *Platythyrea punctata* (Ponerinae) (ventral view), *Wasmannia auropunctata* (Myrmicinae), *Temnothorax sp*. (Myrmicinae). Both representatives of the Formicinae lack visible deposits.



Supplementary Figure S5: Transmission electron microscopy reveals similar external morphologies in queen and worker destined larvae of the ant *Cardiocondyla obscurior*



Supplementary Figure S6: Workers are similarly attracted to queen and worker third instar larvae. (A) number of contacts, (B) duration of contacts



Supplementary Table S1: Crystalline deposit patterns in third instar larvae predict caste in some *Cardiocondyla* species

Species	Clade	Crystalline deposit pattern	larvae with queen-like pattern			larvae with worker-like pattern		
			survival until pupation	queens	workers	survival until pupation	queens	workers
C. wroughtonii	A	queen-like + worker-like	10% (3/30)	100% (3/3)	0% (0/3)	23% (7/30)	0% (0/7)	100% (7/7)
C. nuda	B	queen-like + worker-like	75% (12/16)	100% (12/12)	0% (0/12)	50% (10/20)	10% (1/10)	90% (9/10)
C. venustula	B	queen-like + worker-like	20% (4/20)	100% (4/4)	0% (0/4)	76% (16/21)	0% (0/16)	100% (16/16)
C. thoracica	A	worker-like	-	-	-	80% (8/10)	50% (4/8)	50% (4/8)
C. minutior	B	worker-like	-	-	-	60% (12/20)	8% (1/12)	92% (11/12)

Supplementary Table S2: Overview of ant species screened for crystalline deposit patterns

Species	Subfamily	Crystalline deposit pattern	Source
Acromyrmex versicolor	Myrmicinae	worker-like	www.alexanderwild.com
Adetomyrma goblin	Amblyoponinae	worker-like	www.alexanderwild.com
Amoldius species	Dolichoderinae	worker-like	www.alexanderwild.com
Aphaenogaster picae	Myrmicinae	worker-like	www.alexanderwild.com
Aphaenogaster tennesseenis	Myrmicinae	worker-like	www.alexanderwild.com
Azteca alfari	Dolichoderinae	worker-like	www.alexanderwild.com
Calyptomyrmex piripilis	Myrmicinae	worker-like	www.alexanderwild.com
Camponotus mus	Formicinae	no visible deposits	live colonies
Camponotus sansabeanus	Formicinae	worker-like	www.alexanderwild.com
Cardiocondyla "argyrotricha"	Myrmicinae	worker-like	live colonies
Cardiocondyla elegans	Myrmicinae	worker-like	live colonies
Cardiocondyla emeryi	Myrmicinae	worker-like	live colonies
Cardiocondyla minutior	Myrmicinae	worker-like	live colonies
Cardiocondyla nuda	Myrmicinae	worker-like + queen-like	live colonies
Cardiocondyla obscurior	Myrmicinae	worker-like + queen-like	live colonies
Cardiocondyla thoracica	Myrmicinae	worker-like	live colonies
Cardiocondyla venustula	Myrmicinae	worker-like + queen-like	live colonies
Cardiocondyla wroughtonii	Myrmicinae	worker-like + queen-like	live colonies
Doleromyrma darwiniana	Dolichoderinae	worker-like	www.alexanderwild.com
Formica rufibarbis	Formicinae	no visible deposits	live colonies
Gnamptogenys mordax	Ectatomminae	worker-like	www.alexanderwild.com
Gnamptogenys striatula	Ectatomminae	worker-like	www.alexanderwild.com
Iridomyrmex victorianus	Dolichoderinae	worker-like	www.alexanderwild.com
Linepethima humile	Dolichoderinae	worker-like	live colonies
Monomorium floricola	Myrmicinae	worker-like	www.alexanderwild.com
Myrmecia piliventris	Myrmicinae	worker-like	www.alexanderwild.com
Myrmecia pilosula	Myrmicinae	worker-like	www.alexanderwild.com
Nylanderia faisonensis	Formicinae	worker-like	www.alexanderwild.com
Ochetellus species	Dolichoderinae	worker-like	www.alexanderwild.com
Odontomachus clarus	Ponerinae	worker-like	www.alexanderwild.com
Orectognathus versicolor	Myrmicinae	worker-like	www.alexanderwild.com
Paraparatrechina	Formicinae	worker-like	www.alexanderwild.com
Pheidole dentata	Myrmicinae	worker-like	www.alexanderwild.com
Pheidole desertorum	Myrmicinae	worker-like	www.alexanderwild.com
Pheidole floridana	Myrmicinae	worker-like	www.alexanderwild.com
Platythyrea punctata	Ponerinae	whole body	live colonies
Pseudomyrmex gracilis	Pseudomyrmecinae	worker-like	www.alexanderwild.com
Pseudomyrmex simplex	Pseudomyrmecinae	whole body	www.alexanderwild.com
Pseudoponera succedanae	Pseudomyrmecinae	whole body	www.alexanderwild.com

Sericomyrmex amabilis	Myrmicinae	worker-like	www.alexanderwild.com
Stigmatomma oregonensis	Amblyoponinae	worker-like	www.alexanderwild.com
Strumigenys clypeata	Myrmicinae	worker-like	www.alexanderwild.com
Strumigenys lujae	Myrmicinae	worker-like	www.alexanderwild.com
Strumigenys rostrata	Myrmicinae	worker-like	www.alexanderwild.com
Tapinoma sessile	Dolichoderinae	worker-like	www.alexanderwild.com
Temnothorax sp.	Myrmicinae	worker-like	live colonies
Temnothorax difficilis	Myrmicinae	worker-like	www.alexanderwild.com
Temnothorax rugatulus	Myrmicinae	worker-like	www.alexanderwild.com
Turneria bidentata	Dolichoderinae	worker-like	www.alexanderwild.com
Wasmannia auropunctata	Myrmicinae	worker-like	live colonies
Wasmannia rochai	Myrmicinae	worker-like	www.alexanderwild.com