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1 **A molecular concept of caste in insect societies**

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14 **Highlights**

- 15 • The categorisation of social insect individuals into two distinct castes -
16 reproductive and non-reproductive forms - may be too simplistic:
17 ecology, life-history and level of social complexity generate variation
18 within and among caste
- 19 • The molecular technological advances of the 21st century provide us
20 with an additional empirical base-line for defining and understanding
21 the caste system in eusocial insects.
- 22 • Molecular signatures, combined with behavioural, physiological and
23 morphological traits, may provide a quantitative and objective method
24 for defining and categorising caste.

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39 **Abstract**

40 The term 'caste' is used to describe the division of reproductive labour that defines
41 eusocial insect societies. The definition of 'caste' has been debated over the last 50
42 years, specifically with respect to the simplest insect societies; this raises the
43 question of whether a simple categorisation of social behaviour by reproductive state
44 alone is helpful. Gene-level analyses of behaviours of individuals in hymenopteran
45 social insect societies now provide a new empirical base-line for defining caste and
46 understanding the evolution and maintenance of a reproductive division of labour.
47 We review this literature to identify a set of potential molecular signatures that,
48 combined with behavioural, morphological and physiological data, help define caste
49 more precisely; these signatures vary with the type of society, and are likely to be
50 influenced by ecology, life-history, and stage in the colony cycle. We conclude that
51 genomic approaches provide us with additional ways to help quantify and categorise
52 caste, and behaviour in general.

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54 **Keywords:** social evolution, eusocial insects, genomics, phenotypic plasticity

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77 **Introduction**

78 One of the defining traits of the eusocial insects (ants, some bees and wasps) is a
79 division of reproductive labour achieved through the evolution of dimorphic castes
80 whereby group members specialise in complementary and contrasting behaviours as
81 either queens (reproductives) or workers (non-reproductives). The evolution of queen
82 and worker castes is the key to the ecological success of these insects [1,2]. It is not
83 surprising, therefore, that in the quest to understand how and why eusociality
84 evolves, researchers have focused on revealing the mechanisms, evolution and
85 function of castes [3–6]. A long-debated issue is the equivalent use of the term
86 ‘queen’ and ‘worker’ to describe division of labour across all types of eusocial
87 societies, and specifically: is the concept of caste theoretically relevant [4,7] and
88 empirically meaningful for the simple eusocial societies?

89
90 Biologists categorise castes based on behaviour and physiology (Table 1). However,
91 the last 5 years has seen a burgeoning of data on gene-level differences among
92 caste in a wide range of eusocial Hymenopteran insects. These data provide an
93 objective, quantitative measure of caste differentiation. Here we discuss how recent
94 molecular analyses support the opinion that a dichotomic concept of castes is too
95 coarse a distinction to properly describe and understand cooperation across the
96 spectrum of social complexity. We define a set of data-driven hypotheses on putative
97 molecular signatures of caste for the eusocial Hymenoptera (ants, some bees and
98 wasps) and advocate an approach that unites these measures of genomic variation
99 (the ‘molecular phenotype’; e.g. gene expression, regulation and functionality) with
100 classical measures of phenotypic variation (e.g. behaviour, physiology and
101 morphology). Due to data availability and potentially confounding contrasting life-
102 history features, our analyses here are restricted to the eusocial Hymenoptera (ants,
103 some bees and wasps), although a similar approach could be taken to define
104 hypotheses for other eusocial groups like the termites [8] or eusocial mammals (e.g.
105 naked mole rats). We suggest that the integration of quantitative molecular data with
106 behavioural and physiological traits has much to offer our general understanding of
107 the evolution of caste differentiation and division of labour in eusocial societies.

109 **Defining caste and why it matters**

110 The three key traits of a eusocial species are that it exhibits overlapping generations,
111 cooperative brood care, and division of labour [9,10]. ‘Caste’ is the mechanism by
112 which one of these traits - division of labour - is achieved, and it provides phenotypic

113 variation on which selection can act. The term 'caste' was historically used to
114 describe the fixed, morphologically distinct queens and workers of the complex
115 eusocial species, like the honeybee, where workers are committed to a lifetime of
116 functional sterility (e.g. [11,12]). 'Caste' has since become a more ubiquitous term,
117 used to describe division of labour in all eusocial societies, including those that do
118 not have lifetime, functionally sterile phenotypes. For example, in the space of a
119 decade, E.O. Wilson's use of the term 'caste' changed from one that required
120 'morphologically distinct' traits [3] to a much looser definition whereby individuals are
121 'specialised...for prolonged periods of time'...and...'some kind of additional
122 marker'... which may change with age, and may not involve external morphology
123 [10]. This latter definition includes facultatively eusocial species, where generations
124 are overlapping, there is cooperative brood care, but division of labour (and thus
125 castes roles) can be temporary and distinguished only by behaviour, (e.g.
126 Stenogastrinae wasps; Halictid bees). Currently, the term 'caste' is used as a catch-
127 all term to describe the full phenotypic diversity of division of labour in eusocial
128 insects. However, the applicability of one term (i.e. caste) to describe societal
129 coordination across all species has been questioned and much of the terminology
130 surrounding castes remains misleading [1,9,13–15].

131
132 Determining precisely the traits used to classify individuals into castes has important
133 implications for the study of eusocial evolution. Indeed, the degree to which castes
134 are differentiated is a key characteristic used to infer the level of social complexity a
135 species occupies, and by inference, what stage in the process of social evolution it
136 may represent [6,16]. The importance of this is illustrated by the recent debate over
137 whether all eusocial species are in fact 'superorganisms' and truly represent a major
138 transition in evolution [7]. The key traits used by these authors to determine whether
139 a species is a superorganism are in fact those which are used to define caste:
140 specifically, loss of totipotency and permanent commitment (during development) to
141 a life-time of either reproduction (as queens) or helping (as workers) [7]. This
142 argument takes the concept of caste beyond semantics and a desire to order the
143 disorder of biology: if caste is being used as a benchmark for defining evolutionary
144 processes, we need clearer, more precise ways to categorise and describe it.

145
146 Ambiguity in the use of the term caste is a long-standing problem (Table 1); we lack a
147 consistent set of quantifiable traits for defining and categorising caste. Almost all
148 definitions agree that 'caste' describes **functionally distinct** phenotypes: indeed,
149 this is necessary for a division of labour. Definitions differ on whether castes are

150 **irreversible**, such that individuals show lifetime commitment to a specific role, or
151 flexible, whether a **morphological distinction** is important, or whether **behavioural**
152 **distinction** alone is sufficient. Complications arise for species which traverse these
153 criteria; for example, some swarm-founding Polistine wasps (e.g. *Polybia*
154 *occidentalis*) where caste is apparently irreversible but individuals lack morphological
155 differentiation. Such confusion can be resolved if species are instead defined as
156 those in which caste is fixed during development (this includes all species with
157 irreversible castes, but does not require there to be morphological differences), or
158 whether caste remains plastic throughout life (these are species whose functional
159 roles are 'reversible', and that lack morphological differences). However, without
160 detailed knowledge of the plasticity of castes in a species, this definition is of limited
161 practical use. Moreover, does this mean that species without developmentally
162 determined roles lack 'real castes' [13]? We require a set of traits that are readily
163 quantifiable in order to accurately describe castes, and use this to categorise a
164 species in relation to its level in the evolution of social complexity.

165

166 The heart of the problem in defining castes is that, like most biological systems, caste
167 is a complex phenomenon and boundaries between castes are often messy.

168 Expression of caste traits (as defined in Table 1) varies considerably. Castes can be
169 so different in appearance that queens and workers can appear to belong to different
170 species (e.g. Attine ants; Figure 1). In contrast, other castes are discernible only from
171 behavioural observations and ovary dissections (e.g. *Polistes* wasps; Figure 1). The
172 simpler eusocial societies present the main issue here: they are functionally distinct,
173 but lack lifetime commitment, morphological distinction and developmental
174 differentiation. As a result, some authors have suggested that the simplest eusocial
175 insect societies are better described as 'casteless' and as cooperative breeders
176 [4,7,12]. Further, traits used to define castes may vary within the lifetime of a colony
177 (e.g. *Polistes* foundresses can behave like queens until the first workers emerge, and
178 thereafter behave like workers [18]), and within the lifetime of an individual (e.g.
179 *Bombus* workers can develop ovaries and become unmated reproductives late in the
180 colony's life). Finally, ecological conditions can influence expression of caste traits
181 within and across species: for example, 'worker' traits are only expressed by Halictid
182 bees living in warm climatic regions, with long summers [19].

183

184 An appreciation of the sources of 'messiness' when defining castes is essential.

185 Taking account of how social parameters, ecological variables and molecular
186 processes influence expression of caste traits is required in order to categorise and

187 explain the patterns observed between reproductive and non-reproductive individuals
188 in eusocial insect colonies [13].

189

190 **Molecular signatures as quantifiable traits for defining castes**

191 The 21st century explosion in molecular techniques allows us to scrutinise the
192 concept of caste at the genomic level. We propose data-driven hypotheses for how
193 genomic analyses of phenotypes may contribute towards a more quantitative
194 definition of castes in eusocial insects (summarised in Table 2). These hypotheses
195 fall into three main facets of genomic variation: gene expression patterns, regulatory
196 processes and functionality.

197 **Differential gene expression patterns as molecular signatures of castes**

198 Levels of differential gene expression between castes differ greatly across species,
199 and may be indicative of the level of social complexity. For example, castes in
200 eusocial species with simple societies (e.g. *Polistes*) appear to differ very little in
201 transcription, with less than 1% of detected genes being differentially expressed
202 [22,23]. The low levels of transcriptional differentiation that underlie reproductive and
203 non-reproductive phenotypes in these societies [22–24] reflect the relative lack of
204 caste specialisation and commitment to specific behavioural and/or physiological
205 roles. In these species, the majority of differentially expressed genes (DEGs) are
206 down-regulated in queens compared to workers [22,25,26]. In these societies of
207 highly plastic phenotypes, queens could be classified as ‘shut-down workers’ – i.e.
208 reproductive workers with a reduced behavioural repertoire. In bumblebees (*Bombus*
209 *spp*), reproductive workers show comparable gene expression profiles to queens;
210 however, non-reproductive workers and queens differ greatly in their patterns of
211 transcription with a total of 5316 DEGs between castes, 2817 up-regulated in queens
212 and 2799 up-regulated in non-reproductive workers [27]. The large transcriptional
213 differences between castes in the more complex eusocial societies (e.g. the
214 honeybee *Apis mellifera*) reflect developmentally-determined castes, resulting in
215 individuals which show lifetime commitment to a role and (usually) morphological
216 differentiation [28,29]; queens typically up-regulate more caste-specific genes than
217 workers [27,30]. This pattern of caste-specific expression is established during larval
218 development, where queen-destined larvae up-regulate at least 70% of the
219 differentially expressed genes [28,29].

220

221 The degree of transcriptional differentiation between castes, along with the putative
222 contrasting patterns in the direction of caste-biased expression (i.e. whereby there is

223 a general up-regulation of worker-biased genes in simple societies, but a general up-
224 regulation in queen-biased genes in more complex societies) are likely to be
225 important molecular signatures of caste (Table 2). As such, these traits may be
226 indicative of the level of social complexity, reflecting a hypothetical transcriptional
227 'tipping-point' in species where caste commitment has evolved.

228 **Regulatory mechanisms as molecular signatures of caste**

229 Epigenetic mechanisms control gene expression by differentially regulating genes in
230 response to environmental or genetic cues [31]; they can also limit the plasticity of
231 gene expression, fixing specific transcriptional patterns irreversibly [31–33].
232 Epigenetic canalization is therefore a key hypothesis in the regulation of the inflexible
233 roles found in eusocial insect species where castes are determined (irreversibly)
234 during development [34,35]. An important question, however, is whether the same
235 epigenetic processes regulate caste in the simpler societies, and whether patterns of
236 epigenetic regulation can be useful signatures of caste evolution and sociality
237 [34,35].

238

239 MicroRNAs (miRNAs), 21-23bp RNAs which specifically target mRNAs and control
240 their translation into proteins, are potential epigenetic candidates in the regulation of
241 caste determination [29,36]. In the simple societies of *Polistes* wasps, miRNA-
242 targeted genes show no caste-specific expression between queens and workers [23],
243 potentially reflecting the caste plasticity of these insects. By contrast, in the more
244 complex societies, e.g. bumblebees and honeybees, genes targeted by miRNAs
245 show differential expression between queen and worker-destined larvae [29,36]
246 (Table 2). Caste-specific targets for miRNAs include the ecdysteroids, involved in
247 insect development [29,36], as well as genes related to structural differentiation [29],
248 e.g. *Distal-less* (antennae and proboscis development) and *No extended memory*
249 (imaginal disc-derived wing morphogenesis) [36]. Although data are currently limited,
250 the conservation or differential expression of miRNAs among phenotypes may prove
251 to be useful molecular signatures of caste and play a useful role in identifying the
252 target developmental pathways for gene-specific silencing experiments.

253

254 DNA can be chemically modified by the addition of DNA methyltransferases (DNMTs)
255 which subsequently regulate gene expression; representatives from this group of
256 enzymes are found in all insect orders, albeit with significant variation [34,37,38].

257 Eusocial Hymenoptera show some of the lowest levels of methylation among insects
258 [39], and moreover methylation rates and patterns vary considerably across levels of

259 social complexity [37]. The highly plastic phenotypes of the simple societies, with
260 putatively reversible castes, show only limited difference in brain methylation
261 changes between castes, [22,23]. Species with developmentally-determined castes
262 (which are irreversible) often exhibit high levels of methylation at key genes and
263 these are associated with caste-biased genes [40] (reviewed in [38]) (Table 2).
264 Levels of brain methylation, therefore, may be an indicator of caste differentiation and
265 social complexity [41]. However, the data are inconclusive. At the species level,
266 whole body analyses of methylation found little support for a correlation between
267 methylation and sociality: for example, methylation levels in non-social insects are
268 not consistently lower than social species, and even within the eusocial Hymenoptera
269 there is no clear correlation between methylation levels and social complexity [39,42].
270 One source of variation that may account for the muddy story of the role of
271 methylation in castes and sociality is the tissue analysed and level of analysis; i.e.
272 caste-specific and brain tissue [22,23,40] versus species level and/or whole bodies
273 [8,30,39]

274

275 Chromatin modifications occur via histone post-translational modifications (PTM)
276 [43,44]. The available data suggest that variation in histone modifications strongly
277 correlates with caste-biased genes in ants and honeybees [45,46]. Caste
278 determination during larval development in *Apis* is modulated nutritionally via the
279 feeding of royal jelly; this substance contains a histone deacetylase inhibitor (HDACi)
280 which instigates contrasting pathways in queen and worker-destined larvae [46].
281 HDACi has also been linked to behavioural reprogramming, where it induces an
282 increase in foraging in ant workers [43]. Queens in both ants and bees exhibit the
283 highest levels of histone modifications [45,47], with extensive alterations
284 concentrated in the ovaries; likewise, workers exhibit distinct patterns of histone
285 PTMs [45]. This work on eusocial species with complex societies suggests that
286 histone PTMs could provide a molecular signature of caste (Table 2); however, to
287 date nothing is known about these processes in eusocial species with simple
288 societies. Determining the capacity of a phenotype to return to developmental
289 pluripotency via epigenetic reprogramming [48] may help us define castes by the
290 presence or absence of a molecular marker that imposes commitment on a
291 phenotype.

292 **Functional specialisations as molecular signatures of caste**

293 General patterns of functional enrichment may be useful signatures of caste, and
294 may be especially useful in determining the level of social complexity exhibited by a

295 species. Indeed, the degree to which there is caste-specific functional enrichment
296 appears to depend on the level of social complexity [49–51]. In species with simple
297 societies, there is little or no functional enrichment of molecular processes between
298 castes [22,23]; e.g. as few as 6 significantly enriched Gene Ontology (GO) functional
299 groups were found between castes in the wasp *P. canadensis* [23]. By contrast,
300 significant levels of functional enrichment are found between castes in species with
301 more complex societies [27], and especially those with developmentally determined
302 castes, such as the honeybee *A. mellifera*, where caste-biased genes show
303 significant enrichment for 235 GO functional groups [52].

304

305 Enrichment of specific functional groups, or pathways, could also be a useful
306 indicator of caste. Across all levels of social complexity, worker-biased genes show
307 some level of enrichment for metabolic processes [22,24,53,52]; along with
308 enrichment of cytoskeletal genes (e.g. *actin* and *myosin*) [22,27,52], these patterns
309 may reflect the augmented energy expenditure associated with worker tasks, rather
310 than queen (reproductive) tasks. Stress-response/immunological genes and metal-
311 ion processing genes [24,52,54] which are associated with off-nest behaviours, also
312 present a possible signature of worker-biased gene pathways. Genes relating to
313 transcription and translation are up-regulated in queens of eusocial species with
314 complex societies [52,55]. These pathways are also targets for the epigenetic
315 regulators discussed above, making a compelling case for considering them as a
316 potential caste-specific functional group. However queens, at all levels of social
317 organisation, also express high levels of metabolic genes [52,56], associated with the
318 energetic costs of reproduction. This overlap in functional enrichment between
319 phenotypes, particularly in eusocial species with simple societies where functional
320 speciality is limited [23], makes caste-specific gene pathways difficult to interpret and
321 highlights the importance of utilising a range of molecular signatures (together with
322 physiology and behaviour) to interpret caste.

323

324 **Summary and Conclusions**

325 The use of the term caste in eusocial insect studies requires further clarification due
326 to the diverse spectrum of behavioural, morphological and molecular attributes.
327 Based on behavioural and/or morphological data, current literature uses the term
328 caste as a catch-all to categorise individuals as 'reproductive' or 'non-reproductive',
329 across the broad spectrum of complexity shown by insect societies. In simple
330 societies, gene transcription differs very little between queens and workers [22,23]

331 with the majority of differentially expressed genes being worker-biased [22,26,56],
332 reflecting the metabolic and immunological costs associated with foraging. At this
333 stage, workers are fully able to become queens, but 'switch-off' (or do not activate)
334 their reproductive capabilities, whilst increasing their behavioural repertoire.
335 Epigenetic mechanisms do not appear to be involved at this phase [22,23], though
336 few studies have looked into this in simple societies.

337

338 In species where queens and workers are determined developmentally, differential
339 gene expression between castes is significant [27,30], and there appears to be a
340 general pattern of an over-abundance of queen-expressed genes [27,30]. Worker-
341 biased genes continue to reflect the behavioural range of both on and off-nest
342 activities [52], while queen-biased genes relate to transcription and translation,
343 reflecting the cellular processes involved in reproduction [30,52,55]. Epigenetic
344 processes are also important in complex societies; caste-biased genes are
345 associated with miRNAs and methylation, in particular genes involved in cell
346 development and differentiation [36,57]. Histone modifications could be the most
347 important molecular signature of caste; they define queen and worker development
348 and present caste-specific patterns of expression [45]. However, further work is
349 required on simple societies and the role histone modifications play at this stage of
350 eusociality in order to establish whether these are patterns that persist across levels
351 of social complexity and in different independent lineages of social evolution.

352

353 The next challenge is to obtain quantitative comparisons of molecular signatures in
354 species representing the different stages of eusocial complexity, at different stages
355 across colony cycles and/or experiencing different ecological conditions in order to
356 fine-tune a holistic set of molecular signatures of caste differentiation that can be
357 combined with the classical phenotypic traits of behaviour, physiology and
358 morphology. A holistic approach such as this would also provide an objective way to
359 determine whether caste is an appropriate term to describe division of labour in the
360 simplest insect societies of eusocial insects, or whether they are in fact better
361 described as cooperative breeders. Future work, using comparable methods of
362 transcriptional profiling (see Kennedy *et al.* [58] on how to overcome methodological
363 issues of transcriptomic data) alongside classic behavioural studies, may provide
364 insights into defining the stage of eusocial evolution at which a dichotomy of 'caste',
365 as opposed to a spectrum of gradual phenotypic variation, is appropriate.

366

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375 **Figure Legend**

376

377 **Figure 1:** Integrating hypotheses on putative molecular signatures for caste
378 with classical phenotypic traits. Caste traits vary across the spectrum of
379 eusociality, from facultative/simple eusocial behaviour to obligate/complex
380 eusocial behaviour. Classical traits for defining Queen (Q) and Worker (W)
381 castes include Physiology, Morphology and Behaviour. Molecular traits that
382 may be used additionally to define castes include Gene expression, Gene
383 Regulation and Functionality. Both morphological differentiation and
384 behavioural specialisation increase with increasing levels of social complexity,
385 from facultative and simple societies to obligate and complex societies.
386 Ovarian physiological traits vary between queens and workers: at all levels of
387 social complexity queens have fully mature ovaries and are mated (have a full
388 spermatheca). Workers typically have undeveloped ovaries/are unmated.
389 However, reproductive physiology in workers can change over time: e.g.
390 workers in facultative eusocial species can develop their ovaries and mate at
391 any time. Workers in many obligate eusocial colonies are able to develop their
392 ovaries but are unable to mate; e.g. in queen-right colonies (QR) of
393 bumblebees (*Bombus spp*) workers have undeveloped ovaries but in queen-
394 less colonies (QL), or after a certain stage of colony development, they may
395 activate their ovaries but remain unmated. In the most complex societies of
396 obligate eusocial species workers are sterile; e.g. workers in higher Attine
397 ants lack a spermatheca, and have very regressed ovaries. Molecular
398 signatures may provide additional traits that help refine the categorisation of
399 caste roles. To date, there is support for the following hypotheses: **(1) Gene**
400 **expression:** The degree to which queens and workers differ in gene
401 expression (measured as the proportion of detected genes that are caste-
402 biased) appears to increase with the level of social complexity. Queens in

403 simple eusocial species, like *Polistes*, show a general down-regulation in
404 gene expression (red arrow) relative to W (green arrow); queens and
405 reproductive workers in *Bombus* differ only subtly in gene expression (green
406 arrows, up-regulation of genes), whilst non-reproductive workers are distinct
407 from both (red arrow, down-regulation). Queens in complex societies exhibit
408 more caste-biased genes (green arrow, up-regulation), while there is little
409 difference in gene expression across worker castes (blue horizontal arrow).
410 **(2) Epigenetic gene regulation.** Caste-biased genes may be regulated by
411 epigenetic processes in the more complex eusocial species, with potentially
412 higher methylation levels in worker-biased genes, but higher levels of histone
413 modifications in queens (not shown). In contrast, simple societies appear to
414 lack caste-specific methylation patterns. **(3) Functional enrichment.** The
415 degree of functional specialisation of caste-biased genes appears to increase
416 with social complexity: castes in simple eusocial species show little functional
417 specialisation whilst in complex eusocial species queens are functionally
418 distinct and workers lack queen-biased gene pathways. Photo credits:
419 *Polistes canadensis* (© Emily Bell); *Bombus griseocollis* (WikiMedia Creative
420 Commons; Source, USGS Bee Inventory and Monitoring Lab); *Atta*
421 *cephalotes* (WikiMedia Creative Commons; Author, Sarefo). Ovarian
422 physiology pictures adapted from Mateus [65].

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449 **posit that social behaviour should instead be categorised into two**
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