Eusocial insects as emerging models for behavioural epigenetics

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Abstract | Understanding the molecular basis of how behavioural states are established, maintained and altered by environmental cues is an area of considerable and growing interest. Epigenetic processes, including methylation of DNA and post-translational modification of histones, dynamically modulate activity-dependent gene expression in neurons and can therefore have important regulatory roles in shaping behavioural responses to environmental cues. Several eusocial insect species — with their unique displays of behavioural plasticity due to age, morphology and social context — have emerged as models to investigate the genetic and epigenetic underpinnings of animal social behaviour. This Review summarizes recent studies in the epigenetics of social behaviour and offers perspectives on emerging trends and prospects for establishing genetic tools in eusocial insects.

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doi:10.1038/nrg3787
Published online 9 September 2014

Animal behaviour is best understood as an emergent phenomenon predicated on the dynamic interpretation of sensory stimuli by the brain. The brain's response to sensory stimuli can be modulated by metabolic processes and the endocrine system[1]; however, one of the most remarkable characteristics of the brain is the ability to condition consistent behavioural responses on the basis of prior sensory experience. In other words, the architecture of an animal brain encodes a persistence or memory (which is broadly defined here to include all short- and long-term forms of memory[2]) of transient sensory stimuli that is used to tune the brain's sensitivity to similar stimuli in the future in order to elicit a fitting behavioural response. Decades of research into the molecular processes that underlie the persistence of behavioural responses through memory mechanisms have shown a key role for the regulation of gene expression in neurons, which are one of the major cell types of the brain[2–6]. However, the specific regulatory mechanisms responsible for establishing and maintaining patterns of neuronal gene expression in response to sensory stimuli remain poorly understood, mainly because behavioural responses arise from the integration of stimuli not only from gene-level networks within a neuron but also from networks that integrate the numerous neurons (and non-neuronal glial cells) within a brain[2,6,7].

Over the past 30 years, two general strategies have emerged from the historically independent field of epigenetics to explain the induction and maintenance of phenotypic states through transcriptional regulation of genome-wide gene expression[4,7–9]. The first strategy is the use of specific transcription factor (TF) complexes and regulatory networks that involve feedback loops (that is, *trans* epigenetics), and the second strategy involves alteration of chromatin structure through DNA methylation, histone post-translational modifications (PTMs), non-coding RNAs (ncRNAs) and associated chromatin regulatory proteins (that is, *cis* epigenetics). These two processes are able to provide an explanation for behavioural persistence and plasticity (that is, altering a behavioural response on the basis of new stimuli) by their ability to establish and maintain quantitative control of transcription for long periods of time, either across cell divisions or within terminally differentiated cells such as neurons.

Several animal species have been developed as models to investigate the various aspects of animal behaviour and brain function, including the fruitfly *Drosophila melanogaster* for aggression and reproductive behaviour[10], mice and rats for learning and memory[11], and the zebra finch for neurodegeneration and regeneration[12]. However, these systems have notable disadvantages for genetic and epigenetic studies of behaviour: fruitflies lack key DNA methylation enzymes[13]; rodents often show strong behavioural variation even in standardized environments[14]; and birds can be difficult to maintain in a laboratory[15]. Moreover, none of these organisms are highly social, and they therefore cannot provide suitable

Social behaviours
The interactions among individuals of the same species, for example, collaboration within a well-defined group such as a colony of eusocial insects.

Polyethism
Variation in the allocation of nest-related tasks among individuals in a colony. Polyethism typically refers to the special case of age-dependent changes in an individual's behaviour (that is, age-dependent or temporal polyethism); however, it more generally denotes differences in behaviour associated with caste (that is, temporal as well as morphological or physical polyethism).

experimental paradigms for the large variety of complex social behaviours seen in other species. By contrast, the species-rich group of eusocial insects (including all ants and termites, as well as some bees and wasps) show many of the most fascinating, complex and enigmatic displays of animal behaviour.

Although individual eusocial insects exhibit behaviours that reflect 'simple' antagonistic or adaptive responses (for example, fight or flight)[16], they also display much more sophisticated behaviours elicited by cooperative interactions among individuals within a social group or a colony setting, such as nursing, foraging, nest maintenance, defence and policing. The regulation of such cooperative colonial behaviours (that is, polyethism (also known as division of labour)) is a central feature of more advanced forms of sociality (BOX 1) and often involves the strict allocation of behaviours to qualitatively distinct groups of individuals (denoted as castes) that may vary by age or morphology. The existence of behaviourally specialized castes in particular underscores the relevance of eusocial insects for behavioural epigenetics research. To this end, key aspects of the brain and the central nervous system, including gross morphology and neuronal connectivity, have been extensively characterized in exemplary eusocial insects, such as the honeybee and some ant species, and have been shown to vary by morphology, age and social context[17–21]. Furthermore, many eusocial insects, especially ants, can be maintained easily and in large numbers in a laboratory while largely preserving their natural social context, which greatly facilitates controlled behavioural analyses (BOX 2).

Polyethism is primarily influenced by two factors: caste morphology and age. Caste morphology is specified during juvenile (that is, larval or nymphal) development for both queen and polymorphic worker castes[22,23]. Within each caste, behaviour also changes in an age-dependent manner, as seen in the classic transition from nursing to foraging in honeybees (*Apis mellifera*)[24,25]. Timing of these behavioural transitions is not necessarily 'hardwired' and is often sensitive to dynamic changes in social context, nutrition and physical environment[26] (FIG. 1a). This intrinsic behavioural plasticity of eusocial insects provides numerous opportunities for experimental manipulation. For example, in honeybees, selectively feeding larvae a protein-rich diet that includes royal jelly induces their development into queens[22,23], whereas removing existing nurses from a colony induces foragers to revert to nursing behaviour[27] (FIG. 1b). Workers of the ant *Harpegnathos saltator* can mate, obtain reproductive status (that is, become gamergates) and function as queens when an existing queen dies or when workers are artificially isolated individually or in groups[28,29] (FIG. 1b). Gamergates can also be reverted to workers both behaviourally and physiologically by temporary separation from a colony (C. Penick, unpublished observations). Thus, insights into the molecular mechanisms that underlie polyethism can be obtained by analysing both natural and artificially induced variation in behaviour among individuals in a colony. In addition, the ecological abundance and diversity of eusocial insect species — which comprise more than 30,000 extant species[30] (see AntWeb) — provide excellent opportunities for comparative studies on the evolution and function of the genetic and epigenetic processes underlying behaviour.

The revolution in next-generation sequencing technologies has greatly accelerated behavioural research in eusocial insects. In 2006, the honeybee *A. mellifera* was the only eusocial insect to feature a draft genome assembly[31]. Subsequently, high-quality draft genomes of two ant species were published in 2010 (REF. 32), which were soon followed by the genomes of five other ant species[33] and most recently by the genomes of the clonal raider ant *Cerapachys biroi*[34], the socially polymorphic sweat bee *Lasioglossum albipes*[35] and the dampwood termite *Zootermopsis nevadensis*[36]. These genomes have facilitated generation of the first epigenomic maps of DNA methylation and histone PTMs in eusocial insects[37–42], and have enabled the field of insect sociobiology to begin to address key molecular questions. Is there a conserved genetic basis for sociality among eusocial insect species[39,43]? What epigenetic processes drive age-, caste- and context-dependent behavioural plasticity[27,40]?

Box 1 | **An overview of insect sociality**

Part of our fascination with social insects stems from their capacity to learn and execute complex decisions not only as individuals but also as integrated social groups[136] — a rare trait in the animal kingdom. Indeed, the best-studied and most sophisticated forms of social behaviour are highly restricted taxonomically and are mainly, although not exclusively, found in the eusocial insect orders Hymenoptera (which includes all ants and some bees and wasps) and Blattodea (which comprises all termites)[22,44]. Eusociality is also found in select species outside insects, including snapping shrimps and naked mole rats.

Eusociality is considered to be the most complex form of sociality and is defined as the cooperative care of offspring, which are born from reproductive individuals but are reared by non-reproductive individuals with overlapping generations within a colony. Eusociality and other types of sociality (such as communal, quasisocial and semisocial sociality) are likely to have derived from a simpler form of subsociality, which is generally characterized as the display of transient parental brood care[137]. Eusociality may be found in simple societies with a few monomorphic members and basic division of labour (for example, *Lasioglossum albipes* and *Polistes metricus*), as well as in complex societies that contain thousands to millions of workers that show highly specialized and polymorphic division of labour (for example, *Apis mellifera*, *Camponotus floridanus* and *Reticulitermes flavipes*) (BOX 2).

In the insect order Hymenoptera, the state of eusociality has evolved more than 10 times since its first advent in the Cretaceous geological period ~150 million years ago. By contrast, eusociality has evolved only once in the equally old (if not older) termite lineage (Blattodea: Isoptera)[44,138]. It was previously assumed that sociality in ants was derived from their wasp-like ancestors, whereas bees constituted an independently evolved eusocial lineage. However, recent phylogenomic analyses indicate that ants, bees and social wasps may share a common ancestor[139,140]. Notably, sociality in hymenopteran lineages has evolved to qualitatively different degrees[137] and has involved lineage-specific adaptations at multiple scales of genomic organization[39].

In Hymenoptera, a typical colony contains haploid males and diploid females, which are grouped into reproductive (queen) and non-reproductive (worker) castes. The worker caste may be divided further into morphological subcastes, such as minor workers and major workers (also known as soldiers). Additional worker subcastes (for example, media and super-soldiers) exist in few ant species, including leaf-cutters (genera *Atta* and *Acromyrmex*) and some *Pheidole* species. Caste and subcaste differentiation normally occurs during larval development, whereas further behavioural differentiation occurs in adults. In an unusual example of reproductive plasticity, workers in some ponerine ant species, such as *Harpegnathos saltator*, can supersede morphological queens by becoming gamergates (that is, mated egg-laying workers recognized as primary reproductives by remaining workers).

Box 2 | Development of genetic eusocial insect models

Reproductive division of labour, which is instrinsic to eusocial insects, presents a unique challenge for the development of genetic lines because, in the vast majority of species, only a few individuals in a colony are capable of reproduction. However, in a few species — such as the parthenogenetic honeybee subspecies *Apis mellifera capensis*[132], the Halictid bee (also known as the sweat bee) *Lasioglossum albipes*[35], the ponerine ant *Harpegnathos saltator*[28] and the doryline ant *Cerapachys biroi*[34] — all individuals are capable of laying female eggs. In addition, a large percentage (~25%) of queen-destined brood can be experimentally produced in the pharaoh ant species *Monomorium pharaonis*[141]. Moreover, in honeybees, queens can be reared by placing embryos in honeycomb cells that are specially prepared for queens[128]. In selecting a species for genetic manipulation, the importance of controlled crosses cannot be overstated (FIG. 3) because it allows sophisticated genetic manipulations to be carried out, as is routinely done in *Drosophila melanogaster* and mice (for example, gene knockout and knock-in, as well as temporally controlled and tissue-specific overexpression using UAS–*Gal4*, FLP–FRT or Cre–*loxP* systems[142,143]). In this regard, *H. saltator* and *M. pharaonis* readily breed with males from the same colony[28,144], whereas *C. biroi* does not mate and all offspring in this species are female maternal clones as a result of thelytokous parthenogenesis[34,107]. Artificial insemination offers an alternative to controlled breeding that enables genetic crosses, and it has been carried out successfully in honeybees, bumblebees and leaf-cutter ants[145–147]. The following table lists characteristics of several eusocial insect species that are suitable for experimental analyses of social behaviour.

Species	Laboratory controlled breeding	Available clones or inbred lines	Availability of resources		Colony size	Individual size	Worker caste polymorphism	Polygyny	Polyandry	Developmental time (egg to adult)
			Genome sequence	RNAi						
Apis mellifera (European honeybee)	Yes*‡§	No	Yes	Yes	10^4	Large*	No	No	Yes	16–24 days
Bombus terrestris (bumblebee)	Yes*§	No	Yes	No	10^2 *	Large*	No, but continuous size range	No	No	~25 days
Lasioglossum albipes (sweat bee)	Yes*‖	No	Yes	No	10^1 *	Medium*	No	No	No	~1 month
Camponotus floridanus (Florida carpenter ant)	No§	No	Yes	Yes	10^4	Medium*	Dimorphic: majors and minors	No	No	~3 months
Cerapachys biroi (clonal raider ant)	No‖	Yes*	Yes	No	10^2 *	Small	No	Yes	NA	~1.5 months
Harpegnathos saltator (Jerdon's jumping ant)	Yes*‖	Yes*	Yes	No	10^2 *	Large*	No	No¶	No	~2.5 months
Linepithema humile (invasive Argentine ant)	No	No	Yes	No	10^6	Small	No	Yes	No	~3 months
Monomorium pharaonis (pharaoh ant)	Yes*	Yes*	No	No	10^4	Small	No	Yes	No	~1.5 months
Pheidole morrisi (big-headed ant)	No	No	No	No	10^2 *	Small	Dimorphic: majors and minors	Yes	Yes	~1.5 months
Pogonomyrmex species complex (harvester ant)	No§	No	Yes	No	10^3	Large*	Dimorphic: majors and minors#	No**	Yes	~1.5 months
Solenopsis invicta (red imported fire ant)	No	No	Yes	No	10^4	Small	No, but continuous size range	Yes§§	Yes	~2 months
Polistes metricus (paper wasp)	Yes*‡	No	No‡‡	Yes	10^2 *	Large*	No	No	No	~1.5 months
Reticulitermes flavipes (eastern subterranean termite)	Yes*‖	Yes*	No	Yes	10^6	Small	Dimorphic: soldiers and workers	Yes‖‖	Yes‖‖	NA
Zootermopsis nevadensis (dampwood termite)	Yes*‖	Yes*	Yes	No	10^3	Large*	Dimorphic: soldiers and workers	Yes‖‖	Yes‖‖	NA

NA, not available; RNAi, RNA interference. *Features that are desirable for genetic manipulation. ‡Rearing conditions cannot be controlled for large colonies or groups of colonies as easily or as precisely as for colonies that can be reared completely in environmentally controlled chambers; may be subject to seasonal environmental effects. §Workers can lay viable haploid male eggs (the exception is parthenogenetic honeybee subspecies *A. mellifera capensis*). ‖Workers can lay viable haploid male and diploid female eggs (all *C. biroi* workers are reproductive and only lay female eggs). ¶True polygyny involving queens is rare in nature; however, colonies can routinely have multiple gamergates. #Applies to *Pogonomyrmex badius* only. **Some species, for example, *Pogonomyrmex californicus*. ‡‡The genome of *Polistes dominulus* has been sequenced. §§Monogynous and polygynous forms exist. ‖‖Colonies are founded by a single pair of queen and king, which is later replaced (after their death) by multiple queens and kings.

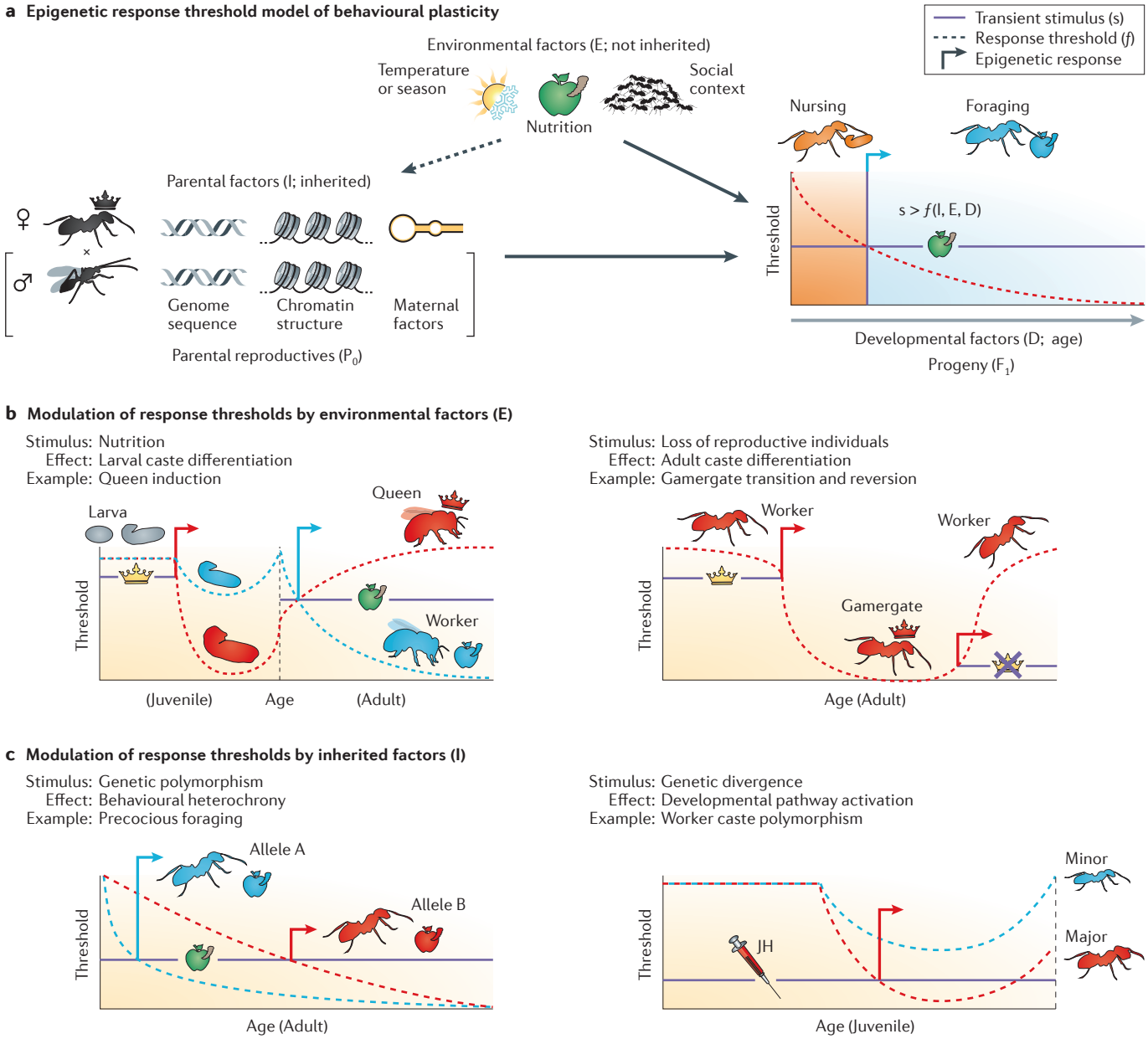

Figure 1 | **Epigenetic response threshold model of behavioural plasticity.** **a** | The phenotypic response of an individual to a transient environmental stimulus depends on several factors: parentally inherited factors (I), which include genotype, epigenotype and maternal factors (for example, maternal mRNA); developmental history (D) that results in its current physiological condition; and biotic and abiotic environmental factors (E). Square brackets around paternal factors indicate that, in eusocial insects, all F_1 males and some F_1 females do not inherit paternal factors from the P_0 generation. The three primary factors (I, E and D) are integrated by an epigenetic transfer function to define a response threshold *f*(I, E, D) that determines an individual's sensitivity to novel environmental stimuli, which are first perceived (for example, by antennal olfactory receptors), and then transduced and 'quantified' into a signal or stimulus through intracellular signal transduction pathways (for example, by neuroendocrine pathways regulating Juvenile hormone (JH) titre (BOX 3)). Only the environmental factors with an internalized signal value (s) that exceeds the response threshold value *f*(I, E, D) can induce stable epigenetic changes to behavioural phenotypes. In the plot, the threshold for foraging behaviour decreases with age (red dashed line). When the foraging stimulus (apple) exceeds the threshold level, the animal undergoes an epigenetic behavioural state transition from nursing (orange) to foraging (blue). **b** | A response threshold changes according to environmental factors (including nutrition), for example, to permit regulation of caste fate in larvae (that is, transient periods of JH sensitivity) (left panel). In many cases, the quantification of the internalized signal value (s) changes rather than the threshold itself; notably, the JH titre decreases through larval stages until it is less than the level needed to prevent pupation. A response threshold changes in adults according to changes in reproductive social context, which results in reproductive polyethism. For example, loss of existing reproductive individuals in *Harpegnathos saltator* induces a transition from worker to gamergate, whereas an induced loss of status leads to a reversion to worker status (right panel). **c** | As physiological condition is determined by the interactions between inherited factors and environmental cues, naturally occurring genetic variation (that is, allelic variation among individuals in a colony) can directly influence response thresholds, for example, by lowering the threshold for a given cue to increase environmental sensitivity (left panel). Genetic divergence may also alter response thresholds in a qualitative manner, for example, by prohibiting sensitivity to a given stimulus (for example, JH), as in the case of major caste determination in dimorphic species or supersoldiers in *Pheidole* sp. ants[130] (right panel).

In this Review, we focus on the latter question through a discussion of how epigenetic processes may regulate behavioural plasticity in eusocial insects by providing the molecular machinery to translate transient environmental cues into stable transcriptional patterns that can be maintained throughout the marked developmental transitions of insects (that is, across cell divisions during metamorphosis and in terminally differentiated cells such as postmitotic neurons). We also discuss the prospects for studying mechanisms of transgenerational inheritance using eusocial insects and for establishing genetic and genomic tools in these species.

Epigenetic stabilization of transcription

Eusocial insects primarily use three types of environmental factors to control individual phenotype: nutrition, temperature and chemical compounds (for example, pheromones)[22,26,44–46]. These factors are applied to various ends and over different timescales to regulate phenotypic traits such as caste fate (for example, workers feeding royal jelly to queen-destined larvae in *A. mellifera*)[22,23,47], adult reproduction (for example, social dominance in *H. saltator*)[45,48–50] and adult behaviour (for example, age-dependent nestmate interactions in many species)[6,26,51–53]. On the basis of studies in a few eusocial insect species, most notably in *A. mellifera,* it is understood that neuroendocrine signalling pathways — for example, Insulin/Insulin-like growth factor (IGF) signalling (IIS) and Juvenile hormone (JH) signalling — are primarily responsible for transducing these environmental cues (BOX 3). However, it remains unclear how these typically transient cues, as well as subsequent intracellular and intercellular signalling cascades, induce stable behavioural states in the brains of eusocial insects[54].

We propose that combinations of *cis* epigenetic processes (which involve DNA methylation, histone PTMs and ncRNAs) and *trans* epigenetic processes (which involve TFs and ncRNAs) underlie behavioural plasticity by their ability to maintain transcriptional patterns over time. Notably, this mechanism broadly applies to two key aspects of adult behavioural identity: the maintenance of chronological memory of neurotransmission events in postmitotic neurons and the maintenance of inherited memory of cellular identity, which is specified during larval development and transmitted through metamorphosis[3,7]. Such an epigenetic model of behaviour complements our understanding that behavioural states correspond to patterns of synaptic connectivity encoded in neural networks[53], as neuronal connectivity, synaptic plasticity and firing sensitivity are themselves environmentally sensitive cell-specific phenotypes that depend on dynamic non-genetic mechanisms to maintain state. Consistent with this model, regulation of gene transcription by established epigenetic mechanisms has key roles in learning, memory and neuronal development in insects and mammals[55,56]. Although transcriptional regulation is by no means the only key layer of regulation of neuronal gene expression — subcellular mRNA localization and local translation[57,58] also have important and established roles — it is notable that transplantation of mRNA complements between neuronal cell types is sufficient to alter cell type identity[4,59]. This suggests that epigenetic processes that regulate gene expression could provide the major mechanism for the regulation of neuronal memory and therefore emergent properties of the brain, such as animal behaviour. One important nuance to this model pertains to genetic polymorphisms (see below). Any genetic variant that alters an epigenetic response to an environmental cue may consequently affect the likelihood of a particular behavioural response (for example, single-nucleotide polymorphisms (SNPs) that contribute to pollen hoarding in honeybees[60]). In this way, naturally occurring genetic variation in a colony may bias the behavioural repertoires of individuals (FIG. 1a).

TFs and histone acetylation coordinate caste fate in ants. Recent comparative genomic analyses have identified several TFs that are associated with caste fate and behavioural plasticity in eusocial insects[39]. A genome-wide study of nearly 30 solitary and eusocial insect genomes analysed the number of TF binding sites that are found near promoters of orthologous genes, and revealed that evolution of TF binding sites is more divergent among eusocial insects than between solitary and eusocial insects. Genes with the most significant evolutionary changes are enriched for neuroendocrine function, show differential expression between castes in both *H. saltator* and *Camponotus floridanus* ants, and are associated with neuronal-related TFs, such as Cyclic AMP response element-binding protein (CREB), Empty spiracles (EMS) and Grainyhead (GRH). These findings suggest that neuronal gene networks have been targeted for regulatory rewiring in two independent eusocial lineages (ants and bees).

CREB was independently identified in the honeybee, in which mRNA levels of the target genes of CREB were found to vary with age-dependent behavioural states (that is, foraging, maturation and aggression) using a compendium of transcriptome profiles from individual brains[61]. CREB and other TFs — including those involved in Bone morphogenetic protein (BMP) signalling (such as MAD, Medea and Schnurri) and chromatin regulation (such as GAGA factor) — are also associated with caste-specific recruitment of the transcriptional co-activator CREB-binding protein (CBP) in *C. floridanus*[40]. This suggests some degree of coordination between TF (*trans* epigenetics) and chromatin (*cis* epigenetics) regulatory processes, as CBP is the major acetyltransferase for a key histone residue, histone H3 lysine 27 (H3K27), in insects[62] (FIG. 2a). Indeed, changes in H3K27 acetylation (H3K27ac) correlate with changes in both CBP and mRNA expression between major worker and minor worker castes in *C. floridanus*. Furthermore, developmental and neuronal genes show evolutionary increases in the number of CBP-binding sites in *C. floridanus* compared with their orthologues in *D. melanogaster*[40], which mirrors the observation of increased evolutionary variability in CREB-binding sites (see above).

The proposed role for CBP in morphological and behavioural plasticity in ants is consistent with observations in other organisms. For example, CBP is required for long-term memory formation in fruitflies and mice[63,64],

Castes
Specialized behavioural groups within a eusocial colony that often correspond to morphological features and that are generally considered to be a stable, if not permanent, characteristic of an individual. For example, members of the queen caste hatch as adults with wings and can reproduce, whereas members of the worker caste (or castes) are wingless and do not normally reproduce.

Behavioural epigenetics
An emerging multidisciplinary field of research that aims to understand how epigenetic processes transform transient environmental cues into persistent molecular patterns of gene expression in order to modulate animal behaviour.

Queen
A morphologically and/or behaviourally distinct reproductive caste in eusocial insects that often shows specialization in both reproduction and dispersal abilities. Depending on the species, a colony may contain one queen (monogyny) or multiple queens (polygyny). Queens, together with males, constitute the 'germline' of a eusocial insect colony.

Worker
A non-reproductive caste in eusocial insects. Workers cooperatively care for the brood of the colony, forage for food, clean up the nest and defend it against invaders. Workers constitute the body or 'soma' of a eusocial insect colony.

Royal jelly
A nutrient-rich secretion produced by mandibular and hypopharyngeal glands in honeybee nurses, which feed it to larvae to induce their development into gynes (that is, virgin queens).

Gamergates
A unique reproductive caste comprised of mated, fertile workers. In some ponerine species (for example, *Harpegnathos saltator*), gamergates emerge from the existing cohort of workers when a queen dies or is artificially removed from a colony.

and defects in CBP, notably in the histone acetyltransferase domain, cause intellectual disabilities in humans (Rubinstein–Taybi syndrome)[65]. Histone acetylation, including H3K27ac, is a key regulator of transcription and enhancer activity in neurons in fruitflies[66] and mice[3,67]. Therefore, CBP may confer epigenetic memory of gene transcription in neurons and other cell types by maintaining a transcriptionally accessible chromatin environment through histone acetylation and associated Trithorax group protein complexes that contain H3K4 methyltransferase activity, and by antagonizing the activity of Polycomb repressive complexes (PRCs), which catalyse trimethylation of H3K27 (H3K27me3)[65,68].

DNA methylation is linked to morphological and behavioural plasticity in eusocial insects. DNA methylation is a classic epigenetic process that is most widely known to maintain stably repressive chromatin in mammalian X chromosome inactivation and genomic imprinting[65,69], as well as in the developmental silencing of promoters throughout the genome[65,70]. Specific CpG dinucleotides are targeted for methylation either through the recruitment of the *de novo* DNA methyltransferase DNMT3 by TFs or histone modifiers (for example, G9a methyltransferase)[70], or through propagation of pre-existing DNA methylation by the maintenance DNA methyltransferase DNMT1 (REFS 65,70). Unlike in mammals, in which DNA methylation occurs globally and promoter methylation represses gene transcription[71], in most insects — with a possible exception in termites[36] — DNA methylation occurs predominantly over transcribed genes, specifically exons[37,38] (FIG. 2b). In contrast to some insects that lack one (for example, silkworms and red flour beetles lack DNMT3 (REF. 72)) or both (for example, *D. melanogaster*[13,72]) key DNA methyltransferases, DNMT1 and DNMT3 are present and functional in eusocial insects[73], including bees[37,42,74], ants[38], social wasps[41,75] and termites[36,76], as well as in some solitary insects[72] such as parasitoid wasps[77]. These findings have spurred interest in studying potential functional roles of DNA methylation in caste fate and behavioural plasticity.

Indeed, a few recent studies have shown that DNA methylation can regulate both reproductive caste fate and behaviour in eusocial insects. In bumblebees, chemical inhibition of DNA methyltransferase activity promotes worker reproduction in queenless colonies[42]. Consistent with this finding, queen larvae in honeybees have lower genome-wide methylation levels than worker larvae, and knockdown of DNMT3 using RNA interference (RNAi) in worker larvae induces the development of queen-like ovaries in adults, which mimics the effect of royal jelly treatment[78]. This indicates that DNA methylation might be required to inhibit queen development in honeybees, in which case royal jelly may function partly by removing this inhibition. DNA methylation may also be mediated by the IIS pathway, both because royal jelly activates IIS (BOX 3) and because IIS may regulate DNA methylation and histone modification in mammals through the mitogen-activated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K)–AKT–glycogen synthase kinase-3 (GSK-3) signalling pathways[79–82]. Consistent with this hypothesis, royal jelly also contains a functional fatty acid histone deacetylase inhibitor (HDACi)[83] known as 10HDA, which should facilitate gene transcription by increasing chromatin accessibility[65]. In addition, among the *Pogonomyrmex* sp. seed harvester ants (BOX 2), obligately sterile interspecific hybrids show lower levels of global DNA methylation than facultatively sterile conspecific progeny. This suggests that the

Box 3 | The insect neuroendocrine system, IIS signal transduction and JH

The neuroendocrine system is a mechanism involving secreted signals that allows distal cell–cell communication and that is found in all species with a nervous system. The secreted signals, hormones, are typically produced in endocrine glands and regulate a range of cell and tissue types, including neurons, through which they can have a substantial influence on behaviour[148].

Perhaps the best-studied insect neuroendocrine signal transduction pathway involves Insulin/Insulin-like growth factor (IGF) signalling (IIS), which is a conserved target of nutrition-mediated signals[1]. Nutrient uptake is recognized by insulin-producing cells in the brain or fat body, which respond by synthesizing and secreting ligands called Insulin-like peptides (ILPs) into the haemolymph. ILPs are recognized by cognate ILP transmembrane receptors (InRs) that are expressed on all cells and in turn activate the intracellular IIS pathway, leading to altered gene regulation. Overall, IIS has been implicated in the regulation of morphology, behaviour and ageing in eusocial insects[101]. However, the effect of IIS on caste fate is perhaps best understood in the honeybee, in which nurse workers synthesize, secrete and feed royal jelly to queen-destined larvae, hence establishing long-term differences in physiology, behaviour and reproduction in the adult queen. In addition to containing nutritional signals that activate IIS, one of the key functional components of royal jelly is Royalactin (also known as Major royal jelly protein 1) — a 57 kDa protein that activates the Epidermal growth factor receptor (EGFR)[47], which in turn seems to activate a common target of IIS, the InR substrate (IRS)[149].

Recent studies have shown a functional link between IIS and the insect neuroendocrine system through Juvenile hormone (JH)[150]. JH is synthesized in the corpora allata, which is a pair of tissues in the insect brain that function as endocrine glands. JH has a well-characterized role in regulating worker behaviour in honeybees, and this seems to be conserved in ants[29]. For example, treatment of larvae from several species of the dimorphic myrmicine genus *Pheidole* with methoprene (which is a chemical analogue of JH) is sufficient both to induce major-destined workers and to activate a conserved but latent developmental trajectory that expresses a super-soldier caste[130] (FIG. 1c). Similarly, application of methoprene to larvae induces development of majors in the formicine ant *Camponotus floridanus* (D.F.S., unpublished observations) and development of queens in many ant and bee species, including *Apis mellifera* and *Harpegnathos saltator*[22,151].

The methoprene-tolerant (*Met*) gene encodes a well-characterized transcription factor (TF) and JH receptor[152]. A recent transcriptome analysis revealed that many IIS and JH targets are differentially expressed in the brain[6,101], and further functional analyses suggested that the TF Ultraspiracle (USP), which dimerizes with the Ecdysone receptor, may mediate the effects of JH in the brain[153]. Among the known targets of JH is the well-studied egg yolk precursor protein Vitellogenin (Vg), the expression of which has been closely linked to reproduction and fertility in various insects[152,154,155]. In eusocial insects, Vg has a key role in both queen reproduction and worker behaviour[156]. Interestingly, this dual role of Vg involves an inversion of the regulatory relationship between JH and Vg. At the late pupal stage in honeybee development, JH abundance correlates positively with Vg to promote reproduction, whereas in adults JH functionally inhibits Vg as the level of JH increases with age from nurses to foragers[154]. Indeed, knockdown of Vg accelerates the transition to foraging in honeybees[123]. There is also evidence that both IIS and JH may be inhibited by Vg, which suggests that behavioural regulation by this pathway may involve negative (that is, stabilizing) feedback[101,155]. Unlike honeybees and ponerine ants (for example, *H. saltator*), the genomes of which have one *vg* gene locus, multiple *vg* paralogues have been identified in myrmicine ants (for example, *Pogonomyrmex barbatus*) and dolichoderine ants (for example, *Linepithema humile*). Expression analyses of these paralogues suggest that functional divergence of Vg is linked to behavioural specializations specifically in more advanced ant species[157].

Parthenogenetic
Pertaining to parthenogenesis, which is a form of asexual reproduction that produces viable embryos from eggs without fertilization by sperm — notably, haploid male production in Hymenoptera. In some parthenogenetic insects such as *Cerapachys biroi*, female reproductives can lay diploid eggs by thelytoky, thereby producing clonal female offspring.

Thelytokous
The parthenogenetic production of female offspring from unfertilized eggs.

regulation of worker caste fate involves an interaction between genetic factors and DNA methylation[84].

Given these striking results, it is perhaps surprising that genome-wide analyses have found relatively few differentially methylated regions in the brains of age-matched adult queens and workers[27,37]. Similarly, comparison of whole-body DNA methylation patterns in two ant species revealed very few changes between worker castes in *C. floridanus*, and between workers and gamergates in *H. saltator*[38]; however, substantial changes were found between males and females, between workers and queens, and even between virgin and mature queens[38]. These results suggest that, unlike its role as a fairly stable epigenetic mark in mammals, DNA methylation may be used in a more dynamic manner in eusocial insects, in which it changes with developmental stage, age and social context. In support of this view, a comparative analysis of honeybee heads between worker and queen castes at larval and adult stages identified nearly fivefold more differentially methylated genes (DMGs) in larvae than in adults, and a larger proportion of DMGs were upregulated in the worker larvae than in the queen larvae[85].

Analyses of DNA methylation in the brains of honeybees that are engaged in nursing and foraging tasks identified several dozen genes with expression levels that changed inversely with DNA methylation status between behavioural groups[27]. Moreover, foraging bees that were experimentally reverted to nursing recapitulated many of the nursing-specific DNA methylation patterns, which affect genes involved in learning, axon migration, transcription and translation. Given the involvement of DNA methylation in learning in mammals[86], methylation patterns associated with foraging in eusocial insects may facilitate learning or memory[72]. Interestingly, genes involved in the regulation of chromatin accessibility[27] and JH signalling[85] also showed differential DNA methylation, which suggests that DNA methylation may target genes with pleiotropic regulatory effects. In addition to regulating gene expression, DNA methylation also seems to regulate alternative splicing. Significant correlations between DNA methylation and alternative splicing were reported in honeybees, ants and termites[27,36–38] (FIG. 2b), and RNAi knockdown of DNMT3 in honeybees led to some changes in gene splicing patterns[87]. By contrast, no

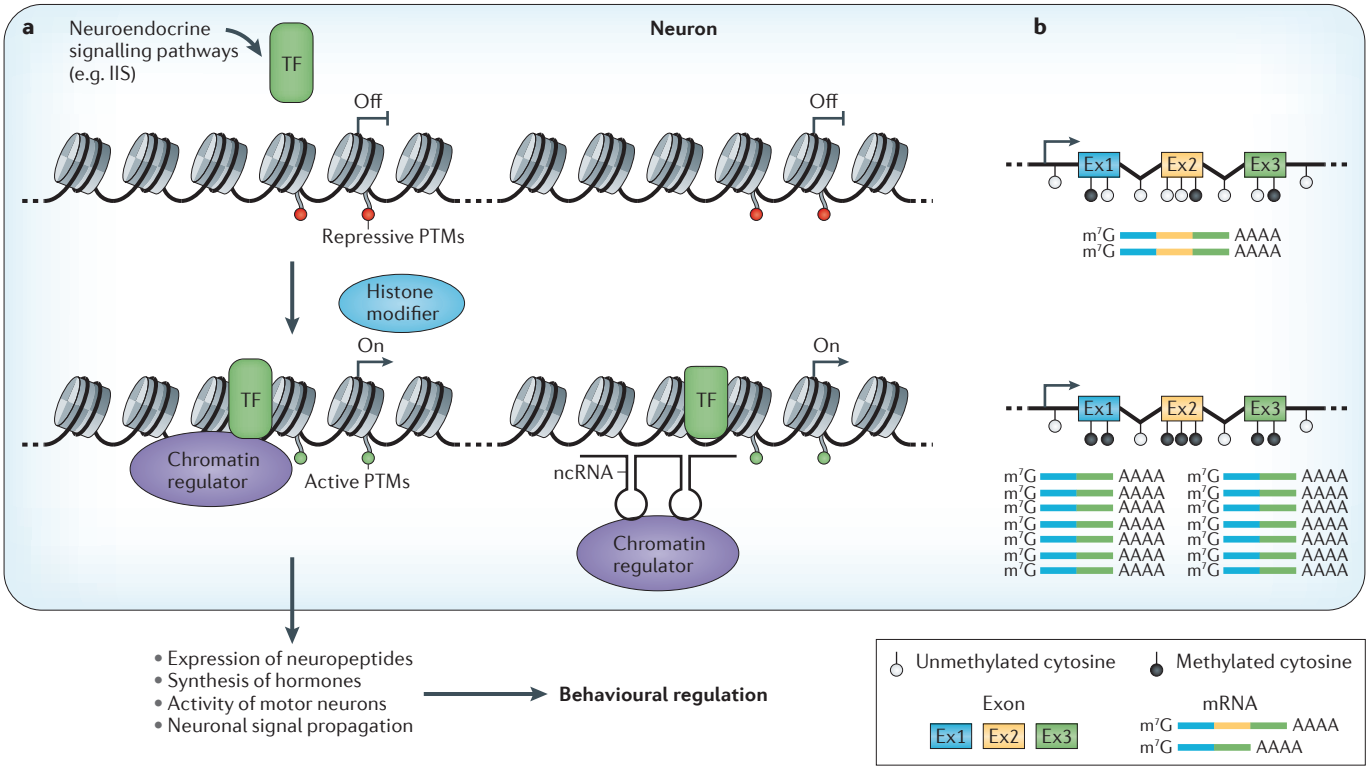

Figure 2 | **Epigenetic mechanisms of gene regulation in the insect brain. a** | The neuroendocrine system regulates gene expression in the brain by targeting transcription factors (TFs), non-coding RNAs (ncRNAs) and chromatin regulatory proteins, which involves histone post-translational modifications (PTMs) and chromatin remodelling. TFs (such as Cyclic AMP response element-binding protein (CREB)) and ncRNAs operate partly through recruitment of histone modifiers (a specific class of chromatin regulatory proteins), such as CREB-binding protein (CBP), which is a transcriptional co-activator that has histone acetyltransferase activity. In this way, signalling pathways may alter the chromatin landscape around target genes from repressive (for example, histone H3 lysine 27 trimethylation (H3K27me3; red circles)) to active (for example, H3K27 acetylation; green circles). Here, chromatin is depicted using the 'beads on a string' model, with DNA (black line) wrapped around histone octamers. **b** | Correlation of gene body DNA methylation with gene expression and alternative splicing is shown. At the genome-wide level, hypermethylation predominantly occurs at genes with medium to high levels of transcription. In addition, inclusion of alternatively spliced exons normally correlates with hypomethylated regions. However, in some cases, the opposite is also true, which suggests the recruitment of different factors to different gene loci[38]. IIS, Insulin/Insulin-like growth factor signalling; m^7G, 7-methyl-guanosine cap.

association between DNA methylation levels and splicing patterns has been found in the solitary hymenopteran wasp *Nasonia vitripennis*[77], which indicates the possibility that certain taxonomically restricted functions of DNA methylation may be involved in regulating plastic behaviour in eusocial insects.

Gene regulation by non-coding RNAs. Various types of ncRNAs, including microRNAs (miRNAs), enhancer RNAs (eRNAs) and long ncRNAs, participate in cell fate determination, neuronal plasticity, embryogenesis and disease progression through transcriptional and post-transcriptional gene regulation[7,88]. Conventionally, ncRNAs are divided into short (<200 nucleotides) and long (>200 nucleotides) classes. Both conserved and lineage-specific short ncRNAs, including miRNAs, have been identified in ants and honeybees, and many of these short ncRNAs show caste-specific expression[39,89]. Interestingly, in *C. floridanus* and *H. saltator*, expression of CpG island-derived ncRNAs positively correlates with the expression of adjacent protein-coding genes, many of which have roles in neuronal development[39]. Long ncRNAs share many characteristics with mRNAs, such as a multiexonic structure, polyadenylation and transcription by RNA polymerase II (Pol II); many long ncRNAs also have tissue-specific expression patterns[90]. As several long ncRNAs can interact with both genomic DNA and epigenetic regulators, they may recruit or stabilize epigenetic modifications at specific genomic loci in a similar manner to TFs (although probably in *cis*)[7] (FIG. 2a). Although there has not yet been a comprehensive identification of long ncRNAs in any eusocial insect, two long ncRNAs were recently characterized in the honeybee in association with worker ovary size[91]. These preliminary findings suggest that the classes of short and long ncRNAs might constitute yet another layer of epigenetic regulation of caste identity in eusocial insects.

Genetic effects on behavioural plasticity

Despite the clear role of the environment in regulating caste fate and behaviour, the phenotype of an organism emerges from the dynamic interplay between environmental and genetic factors[21,53,92,93] (FIG. 1a): behavioural responses depend on both nervous system ontogeny and immediate sensory perception; interpretation of sensory cues depends on the expression of specific receptors, such as those involved in olfaction, gustation and vision; and long-term memory in neurons depends on proper recruitment of chromatin regulators such as CBP[94]. In this context, genetic variation in sequences that regulate gene expression (for example, TF binding sites and ncRNAs) can be used to modulate the thresholds that define an individual's developmental or behavioural response to environmental factors[95] (FIG. 1c). Indeed, caste fate in some species, including *Pogonomyrmex badius*, *Cataglyphis cursor* and *Wasmannia auropunctata*, is not sensitive to environmental variation. Such observations of environmentally invariant caste polymorphism led to early speculations[96,97] and several recent demonstrations[92,98] that genetic variation has an important (albeit fairly uncommon) and complementary role in caste fate determination. Furthermore, increased genetic variation within a colony, which ostensibly decreases kin relatedness, is paradoxically associated with more complex social structures. This has led to the conclusion that genetic variation must be more evolutionarily favourable than previously thought[92,98–101].

Many studies on the effects of genetic variation have focused on reproductive caste ratio (that is, queen production)[98]. However, these studies involved colonies with unusual reproductive strategies for eusocial insects, including polymorphic queen castes and interspecific hybridization[102,103]. Other studies have also examined genetic effects on worker behaviour, which is a more quantitative and variable phenotype than caste fate. Perhaps the earliest of such reports linked age-dependent division of labour in workers to allozyme isomorphs in the polyandrous honeybee *A. mellifera*[104]. Different colonies of the ant *Leptothorax nevadensis rudis* were also shown to exhibit differences in the proportion of foraging workers of similar age[105]. More recently, genetic variation due to polyandry was linked to worker caste polymorphism in *Acromyrmex echinatior* leaf-cutter ants[99] and *P. badius* harvester ants[106]. Such variation may not simply be regarded as a functional side effect, as honeybee colonies show a positive relationship between colonial genetic variation and ergonomic efficiency (which is a proxy for fitness that is sensitive to evolutionary selection)[60]. Therefore, worker behaviour is also subject to genetic effects in both ant and bee lineages.

Notably, these examples of genetic effects on caste and behaviour have been attributed to polyandry, which suggests that genetic variation per se is not a requirement for the expression of temporal or morphological polyethism. Instead, it argues that certain polymorphic alleles can predispose or bias the probability that a given larva will develop into a particular caste or exhibit a particular behaviour, and that the representation of these alleles in a colony can be advantageous[107]. Although such consideration of genetic effects may complicate straightforward analyses of the role of environmental and epigenetic effects on behaviour, it remains an important factor that should be examined. To this end, it would be particularly revealing to use a monogamous but dimorphic species, as may be found in the *Camponotus* or *Pheidole* genera (BOX 2), to assess whether genetic variation that is solely due to maternal heterozygosity is also associated with caste fate or worker behaviour[92,108]. Nonetheless, the often-invoked metaphor that dichotomizes nature and nurture should be replaced by an integrated view of nature plus nurture, in which both genetic and environmental factors have complementary modulatory roles in directing the ontogenetic trajectory of an individual[98] (FIG. 1a).

Transgenerational epigenetic inheritance

Kin selection and inclusive fitness theories provide an evolutionary explanation for the success of eusociality[109,110]. Individuals of a colony may help to increase their colony's fitness indirectly by refraining from reproduction in favour of rearing successive generations of their siblings or relatives. However, as these

Major worker
(Also known as a soldier). A large worker produced in some ant species, for example, *Camponotus floridanus* and *Pheidole morrisi*. Major workers are typically aggressive and specialize in nest defence and carrying heavy or large food items.

Minor worker
A small worker produced in some ant species, for example, *Camponotus floridanus* and *Pheidole morrisi*. Minor workers carry out most tasks in the nest, including foraging.

DNA methyltransferase
An enzyme that catalyses DNA methylation. There are two functional DNA methyltransferase (DNMT) classes in metazoa. DNMT1 carries out maintenance DNA methylation using a pre-methylated DNA sequence as a template, whereas DNMT3 is responsible for *de novo* methylation of DNA. DNMT2 was originally defined as a DNA methyltransferase but was later correctly recognized as a tRNA methyltransferase.

CpG island
An intergenic genomic region that contains a greater density of unmethylated CG dinucleotides than expected compared with genome-wide density. CpG islands were originally defined as regulatory regions in mammals. As insects largely lack intergenic DNA methylation, it remains unclear whether CpG island-like sequences are functional in insects.

Allozyme
A variant form of an enzyme that is encoded by a different allele of the same genetic locus. Allozyme analysis was used to infer genetic variation before direct DNA sequencing became widely used.

Kin selection
A form of natural selection that favours the reproductive success of relatives even at a cost to an individual's own survival and reproduction.

Inclusive fitness
The sum of the reproductive fitness of an individual and the indirect fitness received by relatives other than the individual's own offspring that were produced as a result of help from the individual.

non-reproductive workers are functionally sterile and otherwise phenotypically distinct from their parents, reproductive conflicts are expected to arise between parental and sibling genomes. In ants, bees and wasps, such genomic conflicts are exacerbated owing to haplodiploid sex determination, which creates relatedness asymmetries between maternal, paternal and offspring genotypes. One possible resolution of genomic conflicts may be achieved through the parental regulation of offspring phenotypes, such as fertility potential, through mechanisms of transgenerational epigenetic inheritance, for example, imprinting by allele-specific DNA methylation (ASM)[69,111–115] (FIG. 2). By carrying out reciprocal crosses between two genetic backgrounds, recent studies have shown substantial parental effects on worker defence behaviour in *A. mellifera* honeybees[116], as well as on nursing and foraging behaviours in *Linepithema humile* ants[117]. Initial limited support for ASM was found in *C. floridanus* and *H. saltator* ants[38], in which gene expression levels correlated with increased DNA methylation in an allele-dependent manner. However, it was not determined whether the observed ASM was caused by parental imprinting. Parental effects may also be achieved by mechanisms other than ASM, such as differential deposition of maternal RNAs[118], inheritance of parental chromatin state[119] or both[120] (FIG. 1a). Evaluating these possible mechanisms for resolving genomic conflicts may provide a key perspective on the broader use of transgenerational epigenetic inheritance in animals. Moreover, as many epigenetic mechanisms are sensitive to environmental conditions, these studies may also provide new insights into the influence of the environment in generating phenotypic plasticity in both current and future generations[121,122].

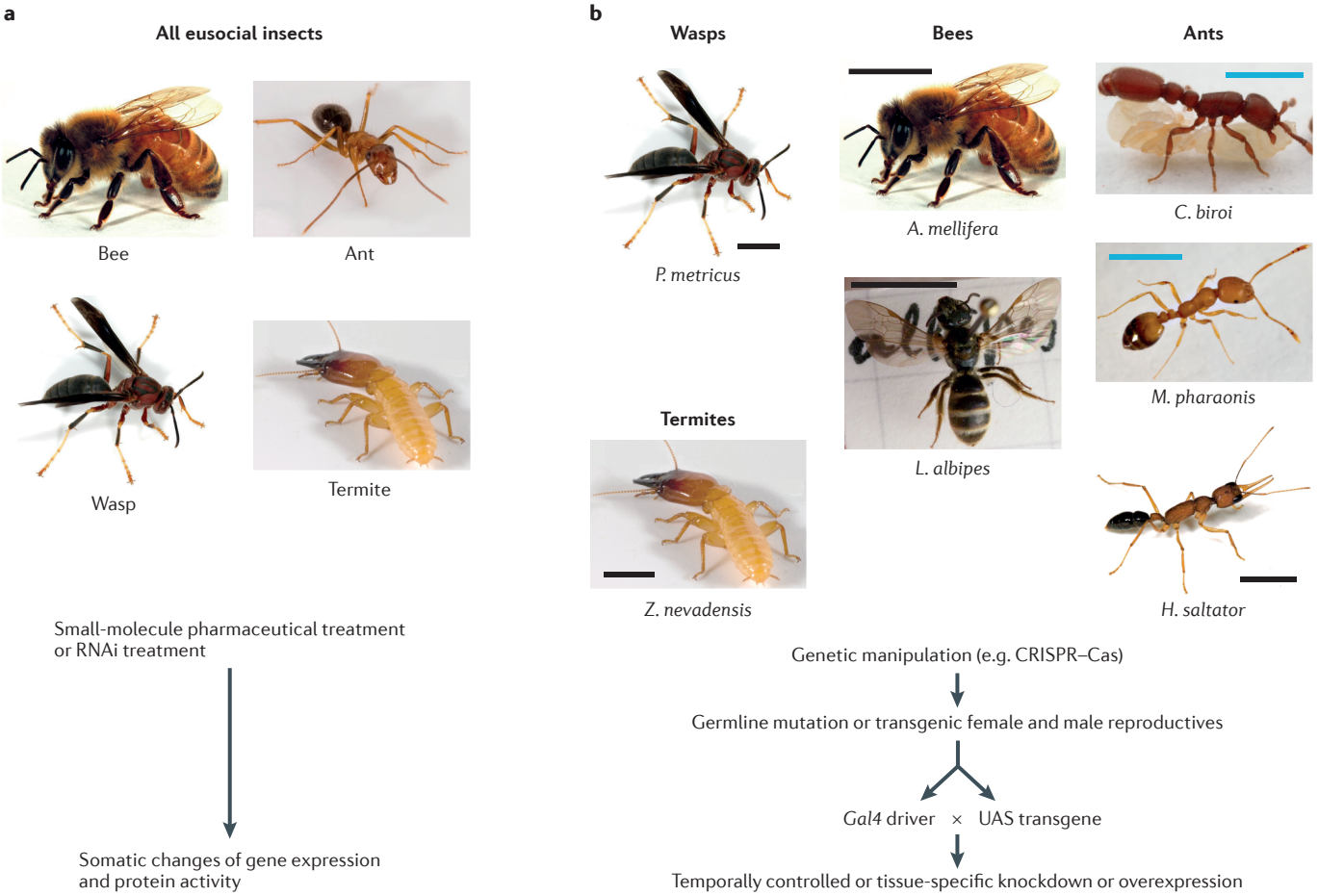

Figure 3 | **Genetic approaches in eusocial insects. a** | Pharmaceutical compounds and RNA interference (RNAi) are currently used to change gene expression in somatic cells to alter caste fate and behaviour in eusocial insects. **b** | A schematic is shown for generating mutant and transgenic lines by germline genetic manipulation in lineages of wasps, termites, bees and ants, such as *Polistes metricus*, *Zootermopsis nevadensis*, *Apis mellifera*, *Lasioglossum albipes*, *Cerapachys biroi*, *Monomorium pharaonis* and *Harpegnathos saltator*. With appropriate manipulation, the reproductive females in these species can either mate or be inseminated artificially to generate progeny from controlled genetic crosses, which are required for some genetic manipulations (for example, temporally controlled or tissue-specific overexpression and knockdown). Blue scale bars represent 1 mm, and black scale bars represent 5 mm. CRISPR–Cas, clustered regularly interspaced short palindromic repeat–CRISPR-associated. *C. biroi* image courtesy of D. Kronauer (The Rockefeller University, New York, USA); *C. floridanus* and *Z. nevadensis* images courtesy of A. Smith (University of Illinois at Urbana-Champaign, USA); *H. saltator* image courtesy of C. Penick (North Carolina State University, USA); *L. albipes* image courtesy of S. Kocher (Museum of Comparative Zoology, Massachusetts, USA); *M. pharaonis* image courtesy of L. Pontieri (University of Copenhagen, Denmark); *P. metricus* image courtesy of P. Coin (Durham, North Carolina, USA).

Haplodiploid
A genetic system of sex determination that is mainly found in the insect order Hymenoptera, including ants, bees and wasps. Hymenopteran females are diploid (that is, they have two complete sets of chromosomes), whereas males are normally haploid and have only one set of chromosomes. Some species, such as the fire ant *Solenopsis invicta*, also produce viable diploid males.

Relatedness asymmetries
Differences in the degree of genetic similarity between parents and offspring that arise in eusocial insects owing to the haplodiploid mode of sex determination and that is exacerbated by polyandry (a form of polygamy in which a female mates with multiple males).

CRISPR–Cas
(Clustered regularly interspaced short palindromic repeat–CRISPR-associated). A technique for generating site-specific mutant or transgenic organisms using the Cas9 protein–guide RNA complex to generate mutations or to direct exogenous DNA to specific genomic regions where it is incorporated.

Eusocial insects as genetic models

Long-term success in addressing the epigenetic basis of eusociality and in establishing eusocial insects as bona fide model organisms will depend on the continued development of genetic and genomic tools (BOX 2; FIG. 3), such as the ability to reduce gene activity using RNAi or pharmacological compounds, or to manipulate genomes and epigenomes through transposon-mediated and gene targeting approaches for mutagenesis and transgenesis (for example, the CRISPR–Cas system). The use of RNAi has been successfully demonstrated in eusocial insects, including honeybees[47,78,123], ants[32,124], social wasps[125] and termites[126]. Pharmaceutical compounds may prove to be especially fruitful to inhibit or activate epigenetic regulators of pathways that are not amenable to RNAi, as the appreciation that epigenetic regulatory proteins have crucial roles in a range of human diseases has led to the development of many compounds that target various conserved pathways[94,127].

The ability to carry out controlled genetic crosses and other straightforward genetic manipulations — historically, the primary consideration for granting the 'model organism' status — remains one of the greatest challenges for many eusocial insect species owing to difficulties in laboratory breeding and inherent limitations in generating large numbers of reproductive individuals. Despite the large sizes of many eusocial insect colonies, which can exceed 10^6 individuals in nature (and at least 10^4 individuals in the laboratory), normally only one to several females can reproduce. However, several specific eusocial species — such as the bees *A. mellifera* and *L. albipes*, the ants *H. saltator*, *C. biroi* and *Monomorium pharaonis,* the paper wasp *Polistes metricus* and the termite *Z. nevadensis* — show a particularly unique potential either for the experimental generation of large numbers of reproductive individuals or for controlled breeding (BOX 2). Indeed, successful generation of transgenic F_1 males has recently been demonstrated in *A. mellifera* by adapting and optimizing techniques that involve piggyBac transposons[128]. This achievement suggests that the first transgenic animals from other eusocial lineages may be imminent.

Conclusion

The recent genomic and epigenomic studies discussed here have provided novel insights into the genetic, epigenetic and molecular underpinnings of social behaviour and evolution of eusocial insect lineages[27,39,40,129,130], and have also offered important molecular interpretations of a response threshold model for colonial division of labour[131] (FIG. 1). Of course, these admittedly few published studies represent only the tip of the iceberg for epigenetics studies in eusocial insects, and the major questions that drive the fields of behavioural epigenetics and sociobiology remain unanswered. What are the principle aspects of a genetic and epigenetic architecture that direct the production of distinct morphological and behavioural castes? What is the extent to which epigenetic modifications, such as histone acetylation[3], drive age-, caste- or social context-dependent behavioural plasticity in brains? Does the evolution of sociality require a distinct genetic toolkit or simply the elaboration and tuning of existing conserved pathways (for example, the IIS–JH, CREB and CBP pathways)? Do reproductive eusocial insects use epigenetic mechanisms to coordinate division of labour transgenerationally in offspring?

A strategic combination of functional characterization, experimental and genetic manipulation, as well as comparison of key genes, pathways and genomes at different scales of biological organization (cells, tissues and colonies), using several taxonomically diverse eusocial 'model organisms' will be crucial to resolve these questions. In practice, bee species such as *A. mellifera* have provided many of the greatest successes in experimental manipulation and breeding, partly as a result of their comparatively long history as ecological and molecular research models[132]. In addition to bees, wasps are increasingly being used as behavioural models that are representative of early stages of eusociality, whereas the variety of ants fully spans the range of eusociality, especially at the most derived and socially complex end of the spectrum. In addition, ants can be reared and bred completely in a laboratory, thereby ensuring the greatest possible control over genetic and environmental variation, in addition to offering substantial advantages in cost, convenience and efficiency. However, many ants have a particular disadvantage in terms of long generation times (BOX 2). Nonetheless, this spectrum of model choices should provide an unprecedented opportunity to understand the roles and interactions of genetic, epigenetic and environmental factors on caste fate, social behaviour and division of labour in eusocial insects. Given the burgeoning interest in eusocial insects (at least as measured by the growing number of related review articles[75,129,133–135]), we envision a rich future for eusocial insects as models for behavioural epigenetics.

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Acknowledgements
The authors thank the three anonymous reviewers and R. Graham for their insightful critiques and suggestions in improving an earlier version of this manuscript. This work has been supported by a Howard Hughes Medical Institute Collaborative Innovation Award (HCIA) #2009005 to D.R., S.L.B. and J.L.

Competing interests statement
The authors declare no competing interests.

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