Current Biology 21, R738–R749, September 27, 2011 ©2011 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2011.06.006

Polyphenism in Insects

Review

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Polyphenism is the phenomenon where two or more distinct phenotypes are produced by the same genotype. Examples of polyphenism provide some of the most compelling systems for the study of epigenetics. Polyphenisms are a major reason for the success of the insects, allowing them to partition life history stages (with larvae dedicated to feeding and growth, and adults dedicated to reproduction and dispersal), to adopt different phenotypes that best suit predictable environmental changes (seasonal morphs), to cope with temporally heterogeneous environments (dispersal morphs), and to partition labour within social groups (the castes of eusocial insects). We survey the status of research on some of the best known examples of insect polyphenism, in each case considering the environmental cues that trigger shifts in phenotype, the neurochemical and hormonal pathways that mediate the transformation, the molecular genetic and epigenetic mechanisms involved in initiating and maintaining the polyphenism, and the adaptive and life-history significance of the phenomenon. We conclude by highlighting some of the common features of these examples and consider future avenues for research on polyphenism.

Introduction

> "*In order to make the term 'polymorphism' more useful and precise, there is now a tendency to restrict it to genetic polymorphism. Since this would leave nongenetic variation of the phenotype without a designation, the term 'polyphenism' is here proposed for it. Polyphenism is discontinuous when definite castes are present (certain social insects) or definite stages in the life cycle (larvae vs. adults; sexual vs. parthenogenetic) or definite seasonal forms (dry vs. wet; spring vs. summer). Polyphenism may be continuous, as in the cyclomorphosis of fresh-water organisms and some other seasonal variation*."
>
> — Ernst Mayr [1]

With this statement in 1963, Ernst Mayr helped return the study of phenotypic plasticity to respectability [1]. During the late 1800s, August Weismann in Freiburg and Edward Poulton at Oxford had shown the power of environmental cues to change the phenotype in moths and butterflies [2,3]. Later, working on helmet length (cyclomorphosis) in clones of *Daphnia*, Leipzig biologist Richard Woltereck [4] introduced the term 'reaktionsnorm' (reaction norm) to describe how the phenotype of an individual depends on the interaction between its genotype and environmental cues. Until publication of Mayr's 1963 book, however, environmentally induced phenotypic plasticity suffered the taint of Lamarckism and was largely ignored in favour of the more respectable, or to use Mayr's words, "more useful and precise" study of genetic polymorphisms, in which phenotypic variants are produced by different rather than the same genotypes [5].

Mayr's definition of polyphenism was broadly inclusive of all manner of discontinuous and continuous phenotypes. Other definitions have been more restrictive. As pointed out by Canfield and Greene [5], however, it is somewhat arbitrary to impose limits such as whether plastic phenotypes are discrete or continuous [6], present within or between developmental stages [7] or seasons [8], fixed rather than reversible [9], or demonstrably adaptive rather than apparently offering no selective advantage. We are inclined to endorse Mayr's more inclusive definition, especially given that what is known of the controlling mechanisms of polyphenisms does not support more restrictive definitional boundaries. As we describe below, this is exemplified by the involvement of the same developmental hormones that control insect metamorphosis in various environmentally induced polyphenisms, including examples that are continuous or discrete in nature. Regardless of the definition used, the insects offer a marvellous array of examples of poylphenisms [10].

Where Does Polyphenism Occur among the Insects?

Everywhere is the brief answer. The developmental stages of insects offer some of the most extraordinary examples, as seen in the transition from larva to pupa to adult in holometabolous (discontinuously developing) insects such as the Lepidoptera (moths and butterflies), Coleoptera (beetles), Hymenoptera (ants, bees and wasps) and Diptera (true flies). Additionally, there are seasonal morphs (exemplified by the aphids and Lepidoptera), density-dependent phenotypes (the defining feature of the group of grasshopper species known as locusts), plastic sexually selected phenotypes (for example, in horned beetles), and diet-mediated phenotypes (as seen in some caterpillar morphs and in the castes of social insects), to mention only a selection [10].

Indeed, polyphenisms are a major reason for the success of the insects. They offer the opportunity for insects to deploy the same genome to partition life history stages (feeding larval stages versus reproducing, dispersing adults), to adopt phenotypes that best suit predictable environmental changes (seasonal morphs) or what might be termed 'predictably unpredictable' environmental shifts such as the transformation of desert environments after unpredictable rain or the degradation of an environment by overcrowding. Insects have even recruited polyphenism to partition labour within social groups, leading to some of the most successful animals on the planet, the eusocial insects.

Here we will leave aside insect developmental stages, and instead focus upon some of the archetypal examples of environmentally induced polyphenisms. In discussing each case we will consider first the nature of the polyphenism, then the sensory cues that trigger shifts in phenotype, the neurochemical and hormonal pathways that mediate the transformation, and finally, the molecular genetic and epigenetic mechanisms involved in initiating and maintaining the polyphenism. Additionally, where it is known, we will discuss

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Figure 1. The catkin (left) and twig (right) morphs in caterpillars of the moth *Nemoria arizonaria* (photo courtesy of Erik Greene).

the adaptive significance and population-level consequences of the phenomenon. As we shall show, no single example is known exhaustively in all of these respects, but each system nevertheless illustrates important aspects of the general phenomenon.

Seasonal and Diet-Induced Morphs in Lepidoptera

Since the work of Poulton and Weismann, the Lepidoptera have provided an abundant source of examples for polyphenism research [11]. Here we will consider two notable examples, the first concerning a remarkable larval polyphenism and the second involving adult butterflies.

The genus *Nemoria* contains over 130 species of geometrid moths, all of which are found in the New World and many of which are superb mimics of their host plants as caterpillars. The species *N. arizonaria* is a host-plant specialist restricted to oak. The moth has two generations each year, one in spring and the other in summer. Caterpillars from the spring generation emerge when the oaks are in flower. They feed upon and develop into perfect physical mimics of oak flowers (catkins). The summer generation, by contrast, emerges onto oaks that are no longer in flower and instead feed upon leaves. These caterpillars grow to look like oak twigs. Adding to the effectiveness of this twig mimicry, when resting they anchor their hind ends and project their bodies outwards at an appropriately twig-like angle (Figure 1).

Greene and colleagues [12,13] showed that the catkin and twig morphs are produced as a result of larval diet: young caterpillars fed catkins grow to be catkin mimics, whereas those fed leaves become twig mimics. Other environmental cues, such as day length, temperature, humidity and background colour, have no effect. Which aspect of food chemistry is responsible for the developmental switch between catkin and twig morphs is not yet known, nor are the physiological and genetic pathways that control the polyphenism.

The second example concerns the southern African butterfly *Bicyclus anynana*, renowned because of the work of Paul Brakefield and colleagues (recently reviewed by Brakefield and Frankino [11]). During the wet season butterflies develop a series of prominent marginal eyespots on the under surface of their hind wings, whereas in the dry season phenotype these spots are greatly reduced and the butterfly is brown in colour, less active and well camouflaged as it sits out the dry months before the rains arrive. Whether butterflies develop into wet or dry season phenotypes is determined by the temperature experienced by larvae in their final stadium: maintaining larvae at 27°C results in the wet season form, whereas at 20°C they develop into the dry season phenotype. The signal initiating the shift in phenotype, however, is associated with the timing of development rather than temperature *per se*. The key hormonal signals are circulating levels of ecdysteroids, whereas juvenile hormone (JH) appears not to be involved [14]. An abrupt shift in the timing of peak concentrations of ecdysteroids in the haemolymph of the pupa occurs at larval developmental temperatures between 21 and 23°C. At 21°C and below, the peak occurs later during pupal development (at 40% of pupal development) than it does at larval rearing temperatures of 23°C and higher (30% of pupal development). Hence, ecdysteroid release translates a continuous environmental cue (temperature) into a threshold phenotypic trait [14].

Experiments involving gene expression studies, use of mutants, and surgical manipulations have uncovered the mechanisms of eye-spot formation (reviewed in [11] and [15]). The initial formation of an eye-spot occurs by activation of a focal region of cells, which signal surrounding epidermal cells to seed the synthesis of pigment, leading to formation of an eye spot. The gene *distal-less* (*Dll*) is involved in stipulation of a focal region [16]. Expression of genes such as *Dll* appears to be regulated via levels of circulating ecdysteroids, but precisely how is not yet known [17].

One of the highlights of research on *B. anynana* has been the experimental analysis of the adaptive significance, quantitative genetics and evolution of eye-spot polyphenism, both in the laboratory and field [11,18–20]. There is strong selection against development of eye-spots in the dry season, largely because they attract avian predators. Selection favouring eye-spot development in the wet season is weaker, although eye spots may help divert predator attacks away from the body under low light conditions [20], and offer some advantage in attracting mates [18]. Quantitative genetics studies in the field, and selection experiments in the laboratory indicate that the reaction norm between eye-spot development and temperature is relatively robust — its amplitude can be shifted but the shape is invariant [11]. Finally, detailed phylogenetic analyses indicate that eye-spot polyphenism has been gained

Figure 2. The solitarious (left) and gregarious (right) forms of the migratory locust, *Locusta migratoria* (photo courtesy of Gabriel Miller).

and lost several times within the genus *Bicyclus* and predates the radiation of the genus.

Density-Dependent Phase Polyphenism in Locusts

One of the most intensively studied and economically significant examples of polyphenism in insects is the density-dependent phase change observed in locusts (Figure 2) [21]. The topic has been reviewed in detail [22–24], but more recent progress has occurred in areas, including: the underlying molecular genetics of phase change [25–28]; the sensory, neural and chemical mechanisms involved [29–32]; the collective behaviour of mass marching locust bands [33,34]; continental-scale population genetics [35–37]; development of biologically inspired practices for managing locust outbreaks [38]; and modelling the adaptive significance and evolution of phase change [39].

The defining feature of the twenty or so species of grasshoppers that are called locusts (see [22] for a list) is that they respond to local population density by shifting between the low-density, cryptic 'solitarious' phenotype (or phase) and the high-density, swarm-forming 'gregarious' phase. Depending on the species of locust, this transition involves a suite of continuously varying traits — including colour, shape, metabolic and hormonal physiology, brain structure, immune function, reproductive life history traits — which do not all share the same underlying mechanisms or time-course [22], but which are effectively coupled as a functional threshold trait by behaviour [23]. Solitarious locusts avoid one another except when seeking mates, whereas gregarious locusts actively aggregate, and at critical densities form marching bands of juveniles (hoppers) or vast winged swarms of adults. The shift from solitarious to gregarious behaviour provides a positive feedback, which, under appropriate environmental conditions, drives phase change at the population level [40,41]. Behavioural phase change occurs in response to crowding within hours. The reverse behavioural transition may either be slower, as seen in the desert locust *Schistocerca gregaria* [42], in which the transition continues across generations via a chemically mediated maternal epigenetic effect [43], or it may occur rapidly as in the Australian plague locust, *Chortoicetes terminifera* [44]. Epigenetic inheritance of other traits, such as colour and morphometry, has also received renewed attention recently in *S. gregaria* [45].

In nymphs of *S. gregaria*, the combination of the sight and smell of other locusts, or tactile stimulation alone is sufficient to induce behavioural gregarization [46], with the hind legs being the principal sites for detecting tactile stimulation [47]. Intriguingly, in the Australian plague locust, *C. terminifera*, touching of the antennae rather than the hind legs induces behavioural gregarization [30]. That different locust species have similar behavioural responses to crowding, but are produced by different mechanisms, reflects the fact that the locusts are a phylogenetically disparate group of species within the grasshopper family (Acrididae); locust-like polyphenisms have evolved independently on more than one occasion, apparently by different means [22].

The neural pathways carrying information about crowding from mechanoreceptors on the hind legs to the central nervous system (CNS) have been identified in *S. gregaria* [48]. Neuromodulation of CNS circuits controlling behaviour is critical for the initial stages of gregarization. A survey of changes in putative modulators within the CNS [49] and subsequent pharmacological interventions revealed that a pulse of serotonin in the metathoracic ganglion, induced either by stimulation of the hind legs or a combination of sight and odour cues from other locusts, is both necessary and sufficient to induce behavioural gregarization in *S. gregaria* [29]. Experiments on the migratory locust, *Locusta migratoria*, involving genome-wide gene expression profiling, RNA interference (RNAi) and pharmacological interventions, have implicated both dopaminergic and serotinergic pathways in behavioural phase change in this species [28]. Dopamine has also been found to differ with phase in *S. gregaria*, but with a longer time-course than that for the initial stages of behavioural change [49]. Whether these locust species differ in the neuromodulatory mechanisms of phase change is yet to be determined, as is the nature and time-course of interactions between the various catecholamine pathways.

Longer-term behavioural changes that are set in train by neuromodulators presumably involve physical changes in neural circuitry, perhaps involving gene expression changes akin to the shift from short-term to long-term memory [29,50]. The study of the molecular genetics of locust phase change was greatly advanced by development of an EST-based microarray for *L. migratoria* [51,52]. This resource has been used to survey gene expression differences between gregarious and solitarious locusts and has implicated numerous compounds as being involved, including hexamerins, haemocyanins, juvenile hormone binding proteins and heat shock proteins [53], as well as differences in the small RNA transcriptome [54]. Also, similar EST-based surveys of phase differences in *S. gregaria* and *C. terminifera* are well advanced. Most recently, next-generation *de novo* sequencing of *Locusta* transcriptomes has pinpointed 242 transcripts as being phase-related [25].

Thanks to these genomic advances, the list of molecular changes associated with density-dependent phase polyphenism in locusts is growing rapidly, but the challenge remains to attribute functions (if any) to these candidates and to discover their roles in the pathway from initiation to maintenance of phase change. One example where

Figure 3. Horned and hornless males of the dung beetle *Onthagophagus nigriventris* (photo courtesy of Doug Emlen).

this has been attempted is the study of Guo *et al.* [27], who showed that *CSP* (chemosensory protein) genes and a gene called *takeout* are differentially expressed in antennae of gregarious and solitarious nymphs of *L. migratoria*. Studies combining RNAi with olfactory behavioral experiments indicated that these genes are linked to the shift from olfactory repulsion to attraction between individuals during behavioural gregarization and associated with the opposite shift during solitarization.

Phase change occurs within individuals, yet its effects scale-up to the behaviour of groups and populations and may ultimately produce swarms. Habitat structure at local scales affects the likelihood that populations of solitarious locusts will be brought together against their predisposition to avoid one another and hence gregarize [40,41,55]. Once aggregated, gregarious locusts self-organise to produce collective mass movement as a result of spatial alignment among individuals [34,56], with most locusts marching in the same direction, driven in part by the risk of cannibalism [33,57,58].

Whereas the mechanisms of locust density-dependent phase polyphenism are becoming understood at levels spanning genetic and molecular events within the nervous systems of individuals through to continental-scale mass migration, additional efforts have been made to understand the adaptive significance of phase polyphenism, for example in relation to predation, disease pressure, and migration [23,39,58–62]. Another avenue of research is mapping the phylogenetic origins of phase characteristics within the grasshopper lineage [63,64].

Beetle Horn Polyphenism

Beetle horn morphology varies substantially within and among species and sexes [65,66], and serves as a model for studies in ecology, development, evolution and sexual selection (for example [67–71]). The study of male horn polyphenism in the dung beetle genus *Onthophagus* (Scarabidae) has proven to be particularly instructive.

Male *Onthophagus* beetles show body-size-dependent expression of horns (Figure 3), whereas females are always hornless. Larger male larvae develop into long-horned adults, whereas smaller male larvae that fail to reach a critical body size typically become beetles with reduced or absent horns. The resulting male horn dimorphism strongly influences mating strategy. Female beetles dig tunnels beneath dung piles, within which they feed, mate and provision progeny at the bottom of the tunnels with balls of dung called brood balls. Males fight for access to females using their horns as weapons. Long-horned males guard tunnel entrances against entry by other males and use their horns as weapons in fights. Smaller males, on the other hand, often employ ‘sneaker male’ tactics: rather than fight, they gain access to females either by sneaking past larger males guarding tunnel entrances, digging alternative access tunnels, or waiting at the entrance to mate with females coming to the surface for food [72,73]. Horn size is an excellent predictor of the outcome of fights, with larger horned males usually victorious. Conversely, the lack of horns in smaller males seems to facilitate manoeuvrability in tunnels in the beetle’s quest to gain clandestine matings [73]. The expression of alternative horn morphologies and mating tactics in *Onthophagus* males is thus a phenotypic response involving multiple traits that vary between the different morphs [74–77]. Whether these various traits have shared or separate regulatory mechanisms remains unknown.

The critical role of nutritional resources in the expression of *Onthophagus* male horn polyphenism was first established in studies by Emlen [78,79]. The nutritional environment experienced by a developing larva is entirely dependent upon the resources provided by its parents in the single brood ball buried by the female. Females deposit a single egg on each ball, which must then sustain the emerging larva throughout its development until pupation. The size and quality of brood ball affects a larva’s growth, body size and conditional horn development [80–84]. In contrast to many other holometabolous insect species, metamorphosis in these beetles is not triggered upon reaching a threshold larval body size; rather, larvae continue to grow until they run out of food, whereupon pupation is triggered by starvation [85,86]. Accordingly, the natural range of variation in brood ball sizes (and presumably nutritional qualities) accounts for the continuous distribution of body sizes in both males and females in field populations. This continuous distribution of body sizes is translated into the discontinuous, bimodal distribution of horn lengths in males by the critical size threshold for horn development [84].

Developmental hormones, in particular juvenile hormone (JH) and ecdysone, appear to play major roles in mediating the effect of an individual’s body size on horn development [87,88]. During a critical period at the end of the larval feeding period, the epidermal horn precursor cells of relatively large males, which have JH levels below a critical threshold, undergo a burst of rapid pre-pupal growth and subsequently develop as fully formed horns. In small males with higher JH levels, above the critical threshold during the sensitive period, the precursor cells fail to proliferate. The difference between the two morphs is correlated with an earlier ecdysteriod pulse in the hemolymph of smaller male larvae that fail to maintain a critical body weight over several days.

Figure 4. Winged and wingless forms of female pea aphids, *Acyrthosiphon pisum* (photo courtesy of Jennifer Brisson).

Ecdysone is thought to affect the fate of horn precursor cells such that they subsequently undergo only minor proliferation during pre-pupal development. Consistent with such a mechanism, developing female larvae, regardless of their body size, also exhibit an ecdysone pulse at the same time as small males and similarly develop into adults with rudimentary horns [88]. The reasons why large males fail to produce the critical ecdysone pulse remain unknown, but downstream changes in the timing and degree of sensitivity to JH have been implicated as a mechanism for the evolution of novel thresholds that can facilitate rapid phenotypic divergence between populations [89,90], and may also lead to developmental trade-offs that can affect the evolution of plasticity and phenotypic diversity [71,77].

Progress has been made in elucidating some of the genes involved in beetle horn development as well as their differential roles in regulating horn development in closely related species [91,92]. However, little is known as yet about the molecular genetic mechanisms underlying beetle horn polyphenism. Few functional genomic resources for horned beetles have existed for this task until the recent development of EST libraries and microarrays for genome-wide transcriptome analyses [93,94].

Aphids as an Example of Wing Polyphenism

Dispersal polyphenisms are well known in insects. These involve phenotypes with differential dispersal abilities, typically winged versus flightless morphs, developing in response to different environments (reviewed in [95–98]). The ultimate selective pressures favouring the evolution of dispersal polyphenisms involve trade-offs between dispersal and reproductive life history traits and, as with all cases of adaptive plasticity, heterogeneous environments are a necessary precursor. When local environments are relatively stable and support reproduction, individuals can achieve higher fitness by allocating resources to reproduction over dispersal. In the face of deteriorating local conditions, investment in alternative phenotypes capable of dispersing to new habitats may be favoured. Wing polyphenism in crickets has provided some of the best-studied examples of dispersal polyphenisms (reviewed in [98]), and locusts too may be construed as another example, with gregarious individuals representing a migratory phenotype. Here we highlight the most intensively and long-studied example of wing polyphenism: the aphids.

Aphids (order Hemiptera) are a diverse group of small insects with stylet-like mouthparts for feeding on plant phloem. Aphid species have diverse and complex life cycles [99] with many exhibiting cyclic parthenogenesis with alternating asexual and sexual generations [100]. In summer, most reproduction occurs through parthenogenesis, with multiple generations of adult females giving birth to fully formed first-instar larvae (viviparity). Sexual reproduction typically only occurs at the end of the season when cues such as falling temperature or the shortening photoperiod stimulate the production of sexual male and female offspring that mate and produce eggs to overwinter. When present, environmentally determined wing polyphenism occurs in females during the parthenogenetic reproduction stage (Figure 4). In males, which are only produced as part of the reproductive generation, both winged and wingless forms have been found in some species. In those species studied, the male phenotype is genetically determined, with wing expression dependent upon the genotype rather than the environment [101,102]. In other words, according to Mayr's [1] definition, wings are polymorphic in males, but polyphenic in females. The presence of both genetically and environmentally determined mechanisms for the expression of winged phenotypes from the same genome provides a unique opportunity to examine the sex-specific evolutionary transition between genetic and environmental control mechanisms for the same trait within the same animal (reviewed in [103,104]).

Numerous cues influence the expression of female wing polyphenism during parthenogenetic reproduction, including population density, host plant quality, temperature, photoperiod, alarm pheromones and interactions with predators, parasites, mutualists, pathogens and endosymbionts [103,105]. The specific responses vary across species and can also vary among clones within a species (for example, see [106]), thereby providing the variation in gene-by-environment interactions upon which natural selection can act to favour different plastic responses.

Although the environmental influences are well known, how these are detected and trigger a phenotypic response remains largely unresolved. For instance, increased tactile stimulation among conspecifics is thought to mediate the effect of crowding; however, the possibility that chemical

cues are involved has not been ruled out, nor have the receptors involved in detecting the relevant stimuli been identified [107]. A long list of cues have been implicated both within and across species, giving the impression of considerable lability in the involvement of different sensory systems, but it may be that seemingly disparate cues are in fact coupled mechanistically. For example, it is conceivable that tactile stimulation may mediate the effects of reduced host plant quality and responses to predators or alarm pheromones by causing aphids to increase their levels of activity and hence contact one another more frequently [105,107]. Such an effect has been shown for locusts, in which reduced food quality or quantity increases the likelihood of a solitarious population gregarizing by increasing interactions among locusts as they move between food patches in search of a better balanced diet, or become concentrated on remaining food patches [55].

Despite considerable investigation, the downstream regulatory events and processes leading to the development of alternative aphid wing morphs are also not known. All aphids examined appear to be born with incipient wing buds, and wings are basal phylogenetically in the group; the capacity to produce wings thus appears to be the default program [108–110]. The loss of wings as an alternative phenotypic state must therefore involve changes in the regulatory pathways leading to wing formation. A potential role for hormones as a signal in aphid wing development has been investigated in detail since the mid-1950s using a variety of approaches (reviewed in [103]). Particular emphasis was placed on JH and its role in maintaining the morphological similarity of wingless adults to nymphs. Under this model, high JH titres were hypothesized to suppress wing development in adults, but experimental support for such a role has been equivocal. For example, Braendle *et al.* [103] highlighted earlier work which showed that decapitation of females exposed to cues that would otherwise induce production of winged morphs results in their nearly immediate production of wingless progeny [109,111]. These findings and others (see [112]) are inconsistent with the model of JH suppressing wing production.

We are likely soon to see a new burst of breakthroughs on aphid wing polyphenism, courtesy of the genomics revolution. The pea aphid, *Acyrthosiphon pisum*, is the first hemimetabolous (direct developing) insect to have had its genome sequenced [113], adding to previously available EST resources [114,115]. Taking a comparative genomics approach using the available fruit fly and flour beetle genomes, Brisson *et al.* [108] identified orthologous genes responsible for wing development in the pea aphid. They also profiled the expression of eleven genes between morphs across different developmental stages, and identified two paralogs of one gene, *apterous*, that are differentially expressed between the morphs during the first and second stadia. Differential expression during this period of development suggests a role in the resorption of wing bud tissue leading to wingless morphs. These findings add to previous genome-wide transcript profiling studies comparing winged and wingless morphs [116,117], which have shown that large-scale patterns of gene expression between wing morphs correspond across different aphid species, and that the downstream transcriptional patterns are very similar, regardless of whether the wing development is environmentally or genetically determined [104].

In addition to being environmentally determined, aphid wing polyphenism has also been shown to be under epigenetic control. During parthenogenetic reproduction, females can be induced to produce winged progeny by appropriate environmental stimuli; however, this winged next generation will themselves produce largely wingless progeny, even in the presence of wing-inducing stimuli [118]. This observation implies that, even though they are genetically identical to their parents, the winged generation has either become insensitive to wing-inducing stimuli or is unable to develop wings. The possibility that DNA methylation regulates gene expression and phenotypic plasticity in aphids has recently been investigated. The full complement of DNA methyltransferase genes has been identified in the pea aphid genome and the genome itself is methylated, indicating the possession of a functional, vertebrate-like methylation system [119]. In light of the hypothesized, but as yet not convincingly supported role for JH in regulating wing polyphenism, Walsh *et al.* [119] used bisulfite sequencing to examine the methylation status of genes known to be involved in the metabolism and transport of JH. While they failed to find any support for morph-specific differences in methylation status in any of the investigated genes, they rightfully pointed out that tissue-specific methylation patterns have yet to be ruled out.

Reproductive *versus* Worker Caste Determination in Eusocial Insects

Eusocial insects provide some of the most familiar and spectacular examples of polyphenism [120]. Although the defining traits of eusociality — cooperative brood care, reproductive division of labour, and overlapping generations — do not include morphological polyphenisms, the vast majority of eusocial taxa (ants, termites, many bee and wasp species, some thrips, aphids, and one beetle species) do contain morphologically as well as behaviourally distinct castes. In some cases, termed 'polyethisms', castes differ in behaviour but not in morphology [121].

A distinguishing feature of eusocial insects is the loss of direct reproduction in most colony members. Reproductive and non-reproductive castes typically have divergent morphologies [122], and often two or more different morphotypes are found within each of these two categories. Caste polyphenism is at the heart of the extraordinary ecological success of eusocial insects, and has attracted great interest since the 19th century. Here we provide a brief overview of some key aspects of caste determination and differentiation in honey bees and termites (Figure 5), two phylogenetically divergent eusocial taxa. Some recent evidence for genetic caste determination (hence 'polymorphisms', using Mayr's definition) challenges the notion that all social insect castes are examples of polyphenisms.

Honey Bees

Among the eusocial insects, the processes underlying honey bee polyphenisms are the best understood. It has long been known that the environmental stimuli leading to the worker and queen castes are dietary in nature: larvae fed relatively low amounts of royal jelly by nurse bees develop into workers, while larvae fed high levels of royal jelly develop into queens [123]. Following a century of research on the biochemical properties of royal jelly [124], Kamakura [125] recently isolated the first compound known to induce honey bee queen differentiation. The compound, a 57 kDa protein dubbed royalactin, significantly increases body size and

Figure 5. Reproductive poylphenism in social insects.

A queen honeybee (*Apis mellifera*, marked in white) surrounded by her worker offspring (left; photo courtesy of Ben Oldroyd.) Caste polyphenism in the termite *Reticulitermes speratus* (right). Mature colonies of this species typically contain a single king (pigmented individual) with numerous neotenic (secondary reproductive) female queens, which are parthenogenetically produced daughters of the founding queen (which dies relatively early in colony development). Several workers at different larval stages (unspecialized) and two soldiers (defensive head and mandibles) are also shown. (Photo courtesy of Kenji Matsuura.)

ovary development, and shortens developmental times, compared with controls [125]. Royalactin also increases JH titre — which is known to peak during the fourth (out of five) larval stage of developing queens. Constant low JH levels are correlated with development of the worker caste [126]. This pattern contrasts with that of many other insects, in which higher levels of JH result in less adult-like forms (see above for beetles and aphids).

Through a number of elegant RNAi knockdown experiments, Kamakura [125] showed that royalactin acts on the epidermal growth factor receptor, which in turn acts on a number of effectors, including: p70 S6 kinase and target of rapamycin (TOR), to increase body size; mitogen-activated protein kinase, to decrease developmental time; and juvenile hormone titres, which promote ovary development. These results are consistent with some previous studies of queen/worker-specific gene expression (reviewed in [127,128]). For example, Patel *et al.* [129] showed that *TOR* mRNA levels are twice as high in queen-destined third-instar larvae compared with worker-destined larvae, and that RNAi knockdown of *TOR* expression blocks queen development. Other upregulated genes in queen-destined larvae include those associated with metabolism and respiration; worker-destined larvae show higher expression of storage proteins.

The promotion of growth — as indicated by higher expression of anabolic pathways such as the TOR pathway — over storage in queens may be due in part to the need for queens to develop quickly, and emerge before their sister queens. An example is provided by the hexamerins, a group of storage proteins that generally act as a source of amino acids in insect haemolymph, and are also known to bind juvenile hormone in some species [130]. In honey bees, the hexamerin genes *hex110* and *hex70a* are transcribed in a caste-specific fashion in pupal and adult fat bodies, with overall transcription being higher in workers than in queens at larval stage 5. Hexamerin gene transcription is inversely correlated with juvenile hormone titre at this larval stage, which prompted the authors to hypothesize that queen development occurs when JH titre exceeds the binding capacity of hexamerins.

Following the demonstration of a functional DNA methylation system in the honey bee [131], Kucharski *et al.* [132] used RNAi to knock down expression in honey bee larvae of the *dnmt3* gene, which encodes a homolog of a methyltransferase shown to be involved in *de novo* methylation in vertebrates. Reducing *dnmt3* expression resulted in a strong bias towards queen (as opposed to worker) development, compared with a control treatment in which a minority of individuals developed queen-like features (though with underdeveloped ovaries) and the majority developed as workers. These results indicate that *dnmt3* is critical for differentiation of the worker caste.

Because methylated cytosines have an elevated tendency to mutate to thymine via spontaneous deamination, genes that are methylated in the germline are expected to undergo a gradual depletion of CpG dinucleotides and to have lower than expected percentages of this dinucleotide relative to genes that are not significantly methylated. Elango *et al.* [133] surveyed the honey bee genome for genes that show this characteristic depletion of CpG dinucleotides. They identified a strong bimodal distribution in the ratio of observed to expected CpG levels among genes, indicating differential methylation in the germline. Furthermore, genes that are methylated in the germline of honey bees seem to be those that are not differentially expressed between the queen and worker castes. The authors speculate that this may be because genes with a relatively high frequency of CpG are prone to epigenetic modification, not in the germline, but at the larval stage and beyond. These modifications may contribute to the developmental differentiation of queens and workers [133]. A similar bimodal distribution of observed to expected CpG has been found in the pea aphid genome [119]. Thus, of the few examples known, insects with discrete, terminal polyphenisms (aphids, honey bees) show such bimodal distributions, while insects without them (*Drosophila*, *Anopheles*, *Tribolium*) do not.

Lyko *et al.* [134] used high-throughput bisulfite sequencing to examine the 'epigenomes' of queen and worker brain tissues. Some 70,000 cytosines out of a total of 60 million across the *Apis mellifera* genome were found to be methylated, almost three orders of magnitude fewer than the human genome. A total of 5,854 genes had methylated cytosines, almost all of which were located in CpG dinucleotides and within exon sequences. Over 550 of these genes showed significant differentiation in methylation patterns between queen and workers. As these genes are expressed at low or moderate levels across all analysed tissues, the significance of differential methylation on gene expression remains unclear. In contrast to the hypothesis of Elango *et al.* [133] that genes with high CpG content should be more prone to epigenetic modulation, the 550 genes differentially methylated between workers and queens had intermediate levels of CpG densities. However, the possibility that the epigenomes of tissues other than the brain follow the proposed pattern has not been ruled out. Interestingly, CpG methylation in a number of genes, including histones, was found to

be significantly denser near differentially spliced exons. An examination of the expression levels for one of these genes showed that one of the splice variants was highly upregulated in queen brain tissue.

Termites

Termites provide an interesting counterpoint to the eusocial Hymenoptera. In the latter, the larvae are grub-like and helpless, and after the larval stages they pupate; only adult female Hymenoptera forage or work in the nest. Termites, in contrast, lack a pupal stage, their larvae resemble small, wingless adults, and larvae of both sexes are active and work in the nest. Furthermore, termites are diplo-diploid, in contrast to the Hymenoptera, which are haplodiploid, with haploid males and diploid females [122]. In most (~80%) termite species, there is an early developmental bifurcation that leads either to the winged, 'alate' caste, or to the wingless worker (or soldier) caste [135]. In the remaining species, a linear pathway operates, with juveniles retaining greater flexibility. Early pioneering studies on caste determination, including Lüscher's elegant experiments in the 1960s, revealed the importance of pheromones in this process (reviewed in [120]). Only recently has the nature of these compounds begun to be elucidated (reviewed in [136]). Matsuura *et al.* [137] isolated the first ever queen pheromone that regulates caste differentiation in termites. They showed that the volatile compounds n-buytl-n-butyrate and 2-methyl-1-butanol are produced by secondary queens of *Reticulitermes speratus* to inhibit the development of further secondary queen production from nymphs. Intriguingly, the same two compounds are also produced in eggs, and serve to both attract workers, and inhibit reproductive differentiation.

The nature of the pheromones and other factors that influence the development of larvae into either the worker or alate caste remains poorly understood, although their effects on JH titres in developing termites have been studied in some detail [136]. In *Hodotermopsis sjostedti,* development of the alate caste requires constantly low JH titres, while the development of workers requires a low JH titre with a peak around the time of ecdysis [138]. The development of soldiers requires consistently high JH titres.

Gene expression studies in termites have shown that, as in honey bees, hexamerins play an important role in caste determination. In *Reticulitermes flavipes*, RNAi studies suggest that hexamerins modulate JH-dependent moulting of workers to the soldier caste [139]. Presoldier morphogenesis in *R. flavipes* is hypothesized to occur when JH titres exceed the sequestration capacity of hexamerins, while status quo worker-to-worker moults occur when hexamerins can successfully sequester available JH. Larger scale examinations of gene expression, DNA methylation, and associated bioinformatic studies will be facilitated by the anticipated sequencing of a termite genome.

Wing polyphenism is an ancient characteristic in termites. The molecular basis of the wing diphenism between the worker and alate castes has yet to be examined, but may benefit from an approach used by Abouheif and Wray on ants [140]. These authors investigated how gene pathways associated with wing development in four ant species are modified to prevent wing expression in the worker caste. Caste-specific wing polyphenism is also ancient in ants, leading the authors to predict that all species share a common mechanism for wing suppression. Surprisingly, Abouheif and Wray [140] found that the wing formation pathways are interrupted at different points in different species, varying even between the soldier and minor worker caste of a single species. This evolutionary lability occurs despite the highly conserved nature of the wing development network over 300 million years of holometabolous insect evolution. Based on these results, the authors predicted that evolutionary lability and dissociation of gene networks are general characteristics of polyphenism. This prediction has been borne out in recent studies of other hymenopteran eusocial insects [141], and awaits testing in termites.

Genetic Caste Determination in Eusocial Insects

Kin selection theory predicts that any allele encoding sterility should be carried both by queens and sterile workers and should be facultative: that is, conditionally expressed in the latter based on environmental stimuli [121]. If such an allele were only present in sterile workers, it could never be passed on. Empirical studies during the last century provided strong evidence for the role of environmental factors in caste determination [142] and this led to the general view that genotype played little or no role in this process. This view has been challenged over the last decade by the discovery of genetic influences on caste determination in several species (reviewed in [143]). The extent of the influence of genotype on caste determination varies between species, ranging from relatively minor to cases where the caste of an individual is essentially hard-wired by its genotype. Examples of the former include the leaf cutting ant *Acromyrmex echinatior* and two honey bee species. In these species, queens mate multiply and produce a number of 'subfamilies' of workers, each fathered by a different male [144].

If genotype played no role in caste determination, members of each subfamily should be equally represented among developing queens and workers. In contrast to this expectation, some subfamilies are strongly overrepresented among queens, suggesting at least a moderate genetic effect [144]. An example of strong genetic influences on caste comes from southern US populations of the harvester ant *Pogonomyrmex*, which contain differentiated genetic lineages that are derived from a historical hybridization between *P. barbatus* and *P. rugosus* [145]. These lineages always occur in pairs, and queens in each lineage-pair mate multiple times with males of their own as well as with males of the alternative lineage. Inter-lineage offspring develop into workers, whereas intra-lineage offspring develop into queens.

A number of additional cases where genotype and environment have a relatively equal contribution to caste determination are known from termites, stingless bees, and ants [143]. Based on these results, the emerging view is that a continuum exists between purely environmental caste determination and purely genetic caste determination [143]. Most species are expected to fall in the middle of the spectrum, with relatively few at either extreme, so that polyphenism (rather than polymorphism) is still expected to underlie caste determination in most species of eusocial insects.

Perspectives for the Future of Insect Polyphenism Research

It is tempting to think that the flurry of mechanistic discoveries promised, and indeed already delivered in some taxa,

by new and emerging functional genomics (and other '-omics') approaches will enable us to finally close the loop between the environmental cues and developmental processes underlying polyphenisms in insects. However, the results to date suggest that a modicum of caution is warranted. Although the genomics era has provided a wealth of candidate genes and potential regulatory pathways, it has not completely solved the problem yet, in part because of limited genetic resources until recently (bee and aphid genome sequences have helped), but also because the initiating events that trigger downstream regulatory changes are likely to be transitory and localized, as evidenced, for example, by the brief serotonin pulse in the metathoracic ganglion that triggers behavioural gregarization in the desert locust [29]. Finding these initiating events will require properly designed and targeted experiments, rather than sole reliance on exploratory genome-wide expression screens. When conducted, gene expression assays will likely need to be conducted at increasingly higher spatial and temporal resolution [104,146]. In other words, the power of genomics needs to be matched with a sophisticated analysis of the phenotype.

Nevertheless, genomics approaches can be useful in generating hypotheses about what genes play a role in the development of alternative phenotypes. For example, genome-wide expression profiling has implicated some specific genes and pathways as being involved in polyphenisms across a wide range of insects, including hexamerins, vitellogenins, and wing-development and takeout protein genes to name a few. Other potential candidates include heat shock proteins such as Hsp 90, which has been implicated as a 'capacitor' for alternative phenotypes in insects [147,148]. Large-scale gene expression analyses have repeatedly shown environmentally determined gene expression changes to be widespread across taxa ([149], but see [140]). However, establishing cause and effect roles for gene expression in plasticity — genes that cause versus maintain plastic phenotypes — remains an important challenge [146]. Additionally, epigenetic regulatory mechanisms such as DNA methylation are increasingly being implicated in insect polyphenisms, but the interplay between these and other DNA modification mechanisms and the regulation of transcription, post-transcription and translational events remain to be discovered (for example [134]).

Although molecular genetics approaches are likely to play a major role in further elucidating the downstream regulatory pathways involved in generating insect polyphenisms, their utility is clearly being hindered by a lack of information about some of the key steps in the gene–environment interaction. Progress has been patchy in matching an understanding of the relevant environmental signals with the sensory responses and physiological pathways that translate these cues into the resulting phenotype. For example, locusts are the best understood system in which both the stimuli and neural pathways involved in the response to crowding have been characterized, although the response varies across locust species. This is in contrast to the aphid response to crowding, where neither the effective stimuli (as distinct from the associated environmental conditions) nor neural pathways are clearly understood. Similarly, with the exception of recent work in honey bees (for example [125]), the role of dietary or pheromonal cues in mediating polyphenism is ill-defined in eusocial insects and *Nemoria* caterpillars, and the sensory cues that initially trigger differential beetle horn development are not known.

The downstream neural and hormonal pathways that translate environmental cues into plastic phenotypes are reasonably well understood in some instances, including the catecholaminergic modulation of behavioural circuits in locusts. Developmental hormones such as a JH and ecdysteroids are proven to control developmental phenotypes (metamorphosis) in insects, and they are also implicated in all polyphenisms to date, but not necessarily playing a primary causal role as notably seen in locusts and aphids.

How polyphenism evolves, and its consequences at various levels of biological organisation remain ripe for investigation. Candidate routes for the evolution of polyphenism include the adjustment of reaction norms [127], and genetic accommodation [9]. The latter has been demonstrated experimentally in the laboratory [150], but whether it is common in the wild remains to be seen. The types of genetic changes that facilitate the evolution of polyphenic traits from monophenic states are still not well established, but may include slight alterations in control of developmental events, including adjusting their thresholds, sensitivity levels or timing [151,152]. Targeted comparisons of closely related species with and without polyphenisms (such as locusts and related grasshopper species) may shed light on this issue. Related to this question is whether the switch mechanisms that underlie polyphenisms for a certain trait (for example, winglessness) share similarities with those underlying polymorphisms for the same trait in related species, or whether they are unrelated.

Just as accelerated evolution is known to occur in genes with sex-specific expression, genes which show biased or specific expression in one polyphenic form or another might be expected to show elevated evolutionary rates. This is expected due to relaxed selection and reduced pleiotropic constraints. Studies on honey bees [153] and horned beetles [154] have provided some early evidence for this phenomenon, but more detailed studies which include multiple time points for expression, as well as diverse tissue samples, are required to investigate this further. At a broader biological level, the influence of polyphenism on phenomena such as speciation rates and phenotypic diversity remains an issue of key interest. Phenotypic plasticity can provide novel sources of phenotypic variation for selection to act upon in natural populations, while at the same time resulting in trade-offs that can generate a diversity of potential evolutionary trajectories [77].

Acknowledgements

We thank Ben Oldroyd and Toru Miura and two anonymous reviewers for critical review of the manuscript. This work was supported by the Australian Research Council (DP0877542 and DP1097265 and a Laureate Fellowship to S.J.S).

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