Genetic underpinnings of division of labor in the honeybee (*Apis mellifera*)

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Honeybees have been studied for centuries, starting with Aristotle, who wrote the first book about bee breeding. More than 2000 years later, the honeybee entered the genomic era as the first social insect whose genome was sequenced, leading to significant insight into the molecular mechanisms underlying social behavior. Additionally, gene expression studies and knock-down using RNAi have extended the understanding of social interactions. Much of the work has focused on caste determination - the mechanism that results in reproductive division of labor, division of labor within the worker caste, and worker reproduction – an essential process underlying eusociality. Here we review the molecular factors involved in caste determination and the differential regulation of caste-specific genes. Recent findings suggest that division of

labor is influenced by a small number of loci showing high levels of pleitropy, suggesting that changes in a small number of genes lead to large changes in the phenotype.

The evolution of eusociality, the obligate group living of fertile individuals with sterile helpers, is one of the major transitions in evolution [1]. It requires reproductive division of labor, cooperative brood care, and an overlap of generations, and it independently evolved several times, mostly in insects. The striking polyphenism of females resulting in distinct castes with individuals highly specialized in reproduction (queens) and sterile helpers (workers) as well as task specialization resulting in division of labor amongst workers are hallmarks of eusociality.

By far the best-studied eusocial model species is the honeybee *Apis mellifera* (Box 1). With the release of the genome sequence in 2006 [2] (Box 2), the honeybee has been fully established as the main model organism for studying caste determination/differentiation and the genetic basis for division of labor and reproduction. Three main model systems have been used in order to study these fundamental processes: (i) the Cape honeybee, *Apis mellifera capensis*, (ii) "anarchistic" bees, and (iii) colonies derived from a long-term bi-directional selection program for high and low pollen hoarding (Box 3).

Here, we summarize recent developments that enhance our understanding of the mechanisms of caste determination and subsequent changes that underpin social behavior. We focus on four themes: (i) reproductive division of labor, (ii) division of labor between workers, mainly the transition from inside to outside tasks (behavioral maturation), (iii) task specialization of foraging workers, and (iv) worker reproduction. These processes, some of which have a heritable basis in regulatory elements, result in huge phenotypic differences due to networks of genetic interactions as well as the involvement of epigenetics in the form of DNA methylation.

Reproductive Division of Labor - Caste Determination

Generally, diploid eggs of the honeybee can develop into either caste. The fate of an embryo is decided during the third larval instar [3], when the major binary switch occurs that leads to a developmental trajectory resulting in either queens or workers but excluding any intercaste individuals. It is generally assumed that caste is determined largely by the royal larval diet, which provides essential cues for activation of those genes that cause the queen phenotype [4]. This switch is not entirely due to environmental factors, however, as direct genotypic effects have been shown to interfere with caste determination. For example during emergency queen rearing, queens are preferentially reared from specific, but underrepresented subfamilies [5]. The mechanisms by which royal larvae are identified by nurse workers are unknown, but it seems clear that some interaction between nursing workers and the developing larvae are essential to control this intracolonial negative frequency dependent selection.

The major environmental factor influencing caste fate comes from larval nutrition, which differs both in quality and quantity for worker and queen-destined larvae. Queen-destined larvae receive more royal jelly (RJ) [4], a substance produced in the hypopharyngeal glands of nurse bees, which visit queen-destined larvae much more frequently than worker-destined larvae. RJ consists of sugars, vitamins, fatty acids [6], and major royal jelly proteins (MRJP) [7], which are derived from gene duplications of the pigmentation gene *yellow* [8] arranged as a tandem array within the genome. However, independent duplications are found in a variety of social and non-social Hymenoptera indicating that the duplication and potential subfunctionalization is not linked to sociality [9]. A monomer of MRJP1, royalactin, may be a major factor influencing caste, as it degrades with temperature and age of the RJ, and supplementation of degraded RJ with royalactin restores its queen-inducing capacity [10].

However, the roles of the other proteins have not been tested as rigorously, and various studies have shown that a single determination factor may actually be extremely unlikely, as increasing the concentration of sugar in worker jelly increases the number of queens and intercastes produced [11].

Once the queen developmental pathway has been nutritionally triggered, there are well-documented subsequent differences in physiology and gene expression patterns [12,13]. A major difference between queen- and worker destined larvae is the levels of their titers of juvenile hormone (JH), a hormone released by the *corpora allata* that regulates insect development by preventing metamorphosis and thereby ensures growth of the larva. The link between the nutritional input, higher levels of JH in queen-destined larvae [14] and distinct gene expression profiles between developing queens and workers remains a puzzle. Nutrient-sensing pathways including the insulin-/insulin-like signaling cascades and the target of rapamycin (TOR) pathway are prime candidates linking nutrition and subsequent effects on gene expression. The *AmTOR* gene is expressed more highly in queen-destined larvae in the third larval instar relative to worker-destined larvae, but this effect dissipates by the fifth instar. Nevertheless, this stage-specific increase may be critical, as a gene knock-down of *AmTOR* using RNAi in queen-destined larvae resulted in the development of workers [15]. Additionally, an insulin-like peptide (ILP1) and an insulin receptor gene (*IR-2*) are both expressed at higher levels in queens during the second instar compared with workers [16]. A combined knock-down of the insulin-receptor substrate (IRS) and *AmTOR* resulted in the complete abolishment of a JH peak [17] and worker-like development. Ectopic application of JH in double knock-outs (IRS^-/TOR^-) restored the queen phenotype [17], but also results in up-regulation of 52 genes in fourth instar larvae, most of which are also up-regulated in queen-destined larvae [18]. The latter study revealed gene expression differences at the global level (using a cDNA microarray representing 6000 of the 10000 honeybee genes). At the third larval instar differentially expressed genes are more frequent in worker-destined larvae (34

worker-specific vs. 3 queen-specific genes). In the fourth instar this picture changes drastically with more genes showing higher expression in queen larvae (65 vs. 105), many of which are JH responsive (see above). The total number of differentially expressed genes in the fifth instar drops down to 36 [18]. Proteomic studies have shown that there are different proteomic profiles as early as the third larval instar [19] supporting the importance of the time point of the nutritional switch for the caste fate.

Genes with queen-biased expression patterns show a higher evolutionary rate of amino acid substitutions than genes with higher expression levels in workers [20]. This might be the result of the distinct expression of genes within one of the phenotypes thereby reducing effects of antagonistic pleiotropy. Queen-biased genes are more strongly exposed to effects of direct selection whereas worker-biased genes are selected indirectly, which might reduce the selection coefficient acting on these genes (Fig. 1). Using kin-selection theory [21], the different selection pressures on queen- and worker-biased genes (direct vs. indirect) were recently analyzed theoretically [22]. It was shown that queen mating frequency, which also affects the intra-colonial relatedness, is likely to be an important driver of molecular evolution. Their model predicted that high mating frequencies would result in higher rates of substitutions for queen genes than for worker genes, whereas single mating would give rise to similar rates of substitutions for adaptive alleles in both castes [22]. These theoretical predictions are fairly consistent with empirical data on honeybees (multiple mating) [20] and fire ants (single mating) [23].

In addition to their variable evolutionary rates, genes with changing expression patterns during caste differentiation also show differences in the structure of their *cis*-regulatory regions. Genes exhibiting queen-specific expression contain two predominant motifs, whereas 12 *cis*-regulatory motifs [24], which often cluster together or occur in tandem [24], appear in genes with worker-biased expression. Typically these motifs are closely located at the

translation start site, supporting the idea that they play a potential regulatory role [24,25]. Worker specific motifs show similarities to regulatory motifs of *Drosophila* genes, whereas the two queen specific motifs do not show any similarities to other known regulatory motifs suggesting that they are evolutionarily new [24].

However, differences in *cis*-regulatory motifs deduced from bioinformatic analyses alone might not be sufficient to completely explain caste specific gene expression patterns. Epigenetic mechanisms such as histone modifications and DNA methylation may be significantly involved in caste-specific gene regulation. Sequencing the honeybee genome in 2006 revealed that it hosts a complete and functional DNA methylation system [26] in contrast to other sequenced insect genomes outside of the Hymenoptera. Subsequently it was shown that genes that are expressed in brain tissue are more strongly methylated in larvae than in adults and that 80% of the genes with worker-biased expression are methylated [27]. Finally, a knock-out in worker destined larvae of Dmnt3, the enzyme that methylates cytosine residues, results in preferential development of queens or queen-like individuals [28].

Taken together, this work supports the model that caste determination and subsequent differentiation is based on a nutritional switch occurring at the third larval instar that is perceived by the TOR and insulin/insulin-like pathways, which affect hormonal levels (especially JH). The subsequent gene expression profiles result in the upregulation of many JH responsive genes in queen destined larvae in the fourth instar. The nutritional switch for larvae manifests as bi-directional developmental pathways giving rise to distinct phenotypic classes within the female sex (queen- and worker caste), which is the most basic prerequisite for social behavior in social insects. Further, major distinctions can be made for the division of labor within the worker-caste, which we discuss below, especially for the age-polyethism that includes the transition from in-hive to outside tasks.

Division of labor – Nurse to Forager transition

The division of labor among workers (polyethism; e.g. nursing, guarding, foraging) is correlated with the age of the workers. Young workers preferentially perform in-hive tasks and as they age, they move towards the periphery of the nest engaging more frequently in outside tasks [29]. After about 14 days, workers completely switch to foraging, a transition associated with significant physiological changes [30,31]. However, this system shows flexibility for the needs of the colony and is fully reversible, again highlighting the plasticity of phenotypes and behaviors. For example, the removal of foragers results in replacement by young bees as precocious foragers [32]. The regulation of division of labor is a complex interplay of a large suite of factors, both intrinsic and extrinsic. Motivation, experience, physiological state, genotype, local needs, and interactions with other workers all affect the balance of which individuals perform which tasks [33].

Evidence for the influence of the genotype of a worker on the tasks performed by that worker has been found either from QTLs (Tab. 1) correlating with nectar and pollen foraging [34], and stinging behavior [35], or from task specialization of certain subfamilies (due to multiple mating) like undertaking (removal of dead nestmates), guarding behavior at the nest entrance [36], or water collection [37].

One of the major genes involved in the regulation of foraging behavior, *foraging* (*for*), was previously identified in *Drosophila* [38] and is responsible for a distinct foraging and exploration phenotype of *Drosophila* larvae. The naturally occurring polymorphism in that gene segregates in a Mendelian fashion with “rovers”, larvae exploiting the full foraging range, being dominant over “sitters”, which usually show very little mobility during foraging [38]. As the *Drosophila* phenotypes are analogous to the major behavioral classes of honeybee workers (sitters correspond to nurses; rovers correspond to foragers) it has been suggested that the *foraging* gene might be involved in the transition to foraging behavior.

Indeed, gene expression studies showed that *Amfor* is expressed at higher levels in normal foraging workers as well as in experimentally induced precocious foragers [39]. The *Amfor* gene encodes a guanosine 3‘,5‘-monophosphate (cGMP)-dependent protein kinase (PKG), which allows for elegant supplementation experiments to induce the phenotype. Workers treated with cGMP increased their foraging activity whereas cAMP treated workers did not [39]. Thus, the *for* gene in *Drosophila* acts via different alleles, whereas in honeybees the same system is used with a distinct temporal regulation of gene expression. Another gene involved in mediating sensory information during feeding behavior in *Drosophila* is *malvolio* (*mvl*), a manganese transmembrane transporter in the brain influencing the sucrose responsiveness of flies [40]. Expression of this gene is upregulated in foragers compared to nurse bees, and manganese treatment induced precocious foraging and increased sucrose responsiveness [41].

Overall, there are more than 1500 genes differentially expressed in the brains of nurses and foragers [42]. As these behavioral categories are inextricably linked to the age of the workers, experimental manipulations to uncouple behavior from age have been done by using single-cohort colonies resulting in young (precocious) foragers and old nurses. The brain gene expression profiles are much more strongly associated with behavior and only secondarily with age. Individual gene expression profiles robustly predict the behavior performed by individuals with 58 of 60 individuals correctly identified based on the expression pattern of 10-100 of the strongest behavioral predictor genes [42].

The nurse to forager transition is also accompanied by changes in the methylation pattern of genes in the brain. However, if foragers are reverted back to nurses, the methylation pattern will also be reverted back to the pattern typically observed for nurses [43]. This is the first evidence for a reversible change in methylation of genes that is associated with individually performed behavior.

The transition from nurse to forager tasks marks a milestone in the age-polyethism of honeybees. This transition is accompanied by many physiological changes (e.g. reduction in the hypopharyngeal glands in and increases in juvenile hormone titers in foragers) as well as huge differences in brain gene expression profiles. However, the actual pacemaker genes for that transition have not yet been found. Nevertheless, some molecular mechanisms have been unraveled in gene expression patterns of candidate genes associated with the nurse to forager transition that are also involved in an exploratory phenotype (*for*) and in sensing sugar responsiveness (*mlv*) in *Drosophila*.

Division of labor – Pollen vs. Nectar foraging

Once workers have switched to outside work, they may specialize during their foraging trips on the collection of nectar, pollen, water or plant resin. To study task specialization during foraging, an outstanding experiment was started by Robert E. Page in 1990, then at the University of California, Davis, initially using 400 colonies to bi-directionally select for low and high pollen hoarding colonies. Several QTL mapping experiments revealed four QTLs (*Pln1-4*) responsible for increased pollen hoarding [44,45,46]. Although selection was applied at the colony level, genetic and phenotypic effects were detectable at the individual level. These selected strains have been intensively studied with respect to genetics, physiology, and behavior. The QTLs that have been identified show strong pleiotropic effects. For example, *Pln1* and *Pln4* influence the sucrose response threshold (i.e. perception level of sucrose by sensory physiological processes), all four QTLs influence foraging behavior, which as stated above involves over a thousand genes, and *Pln1* affects the age of onset of foraging. Thus, because the bi-directional selection regime for pollen hoarding at the colony level resulted in an array of phenotypic associations, it has been described as the “high pollen hoarding syndrome” [47].

High pollen hoarding (HPH) bees show higher levels of ovary activation coupled with higher gene expression levels for vitellogenin, the major egg yolk protein. A candidate gene within QTL region *Pln2* has been identified as hormone receptor-like in 46 (*HR46*), a nuclear hormone receptor (NHR), which is expressed at higher levels in the low pollen hoarding (LPH) strain during all developmental stages, from larvae to forging workers [48]. By contrast, a major candidate gene within the *Pln3* region encoding a phosphoinositide-dependent kinase-1 (*PDK1*), shows no differential expression in larvae or newly emerged workers, but it does have higher expression levels in HPH foraging workers [48]. *HR46* acts as a co-factor of *βFTZ*-F1, another NHR, to regulate organ size during development, for example by inducing apoptosis in salivary glands of *Drosophila* [49]. These genes might also be responsible for the induction of programmed cell death in worker ovary cells that occurs during pupal development [50]. Higher expression levels of this gene in LPH workers might result in reduced ovary size due to increased cell death during development. By contrast, HPH workers show higher number of ovarioles, larger ovaries, and higher levels of vitellogenin expression [48].

The selection experiment for high and low pollen hoarding colonies has led to colonies showing a behavioral and physiological syndrome that has become the basis for the "reproductive groundplan hypothesis" (RGPH, Box 4). An interacting network of a small number of loci with pleiotropic effects regulates a suite of phenotypes, such as onset of foraging, sugar responsiveness, but also JH titers that in turn affect levels of vitellogenin and hence the degree of ovary development. The analysis of factors contributing to ovary development in functionally sterile workers is important for our understanding of reproductive division of labor and the evolution of a sterile worker caste.

Division of labor – Worker reproduction

Although workers appear to be sterile, they still possess rudimentary ovaries. Nevertheless, worker's ovaries can be activated and develop into fully functional organs under certain conditions. Whenever the queen is lost, the ovary-suppressing effect of the queen's mandibular gland pheromone (QMP), a primer pheromone mainly consisting of 9-oxo-decenoic acid (9-ODA), also disappears. Intra-colonial selection for reproductive dominance among workers [51] by means of pheromonal competition [52] will result in certain workers developing their ovaries and finally laying unfertilized eggs. Bees of subspecies of the Western honeybee differ in their propensity to develop ovaries, suggesting a genetic basis for this behavior. The most extreme cases of ovary activation and subsequent worker reproduction are found in the Cape honeybee, *Apis mellifera capensis* [53] and in so-called "anarchistic" bees (Box 3) [54].

Anarchistic bees were identified in a screen for ovary activation even in the presence of the queen. The phenotype is strongly influenced by genetic factors, and about a third of the workers selected for the anarchistic trait show activated ovaries with oocytes present in the ovarioles at the age of 10 days [54,55]. Associated phenotypic characteristics in lines of anarchistic bees differ substantially from predictions made on the basis of the pollen hoarding syndrome and have been used to question the validity of the RGPH (Box 4) [56]. Anarchistic workers started significantly later with foraging than wild-type workers, but once they started there were no differences to wild-type workers with respect to foraging preferences.

The anarchistic phenotype seems to be a signaling blind mutant phenotype, as workers do not respond to the otherwise effective pheromonal signals emitted by the queen or the brood. Moreover, anarchistic bees are not capable of producing queen-like pheromonal blends as is the case in reproductive workers of other subspecies; it seems that ovary activation in anarchistic bees is decoupled from any other phenotypic characteristic that is usually found in reproductive workers. The fact that it is selectable [57] indicates that there must be a strong

genetic basis for this phenotype. A QTL screen showed that this phenotype is quantitative rather than based on a recessive gene as predicted before [58]. Four QTLs have been identified that together explain 25 % of the phenotypic variation. Some of these QTL regions also contain candidate genes that were identified in an independent cDNA microarray gene expression study [59]. Additional studies using an oligo-nucleotide microarray identified some further genes that are differentially expressed between anarchistic and wild-type workers, resulting in a merged list of 15 candidate genes suitable for further analyses. Most of these genes might not be causative, however, as they are scattered throughout the genome and appear to be unlinked to the QTLs [60].

Interestingly, brain gene expression profiles between anarchistic and wild-type bees do not differ significantly [59], and only a few genes exhibit a differential expression pattern. This is in stark contrast to brain gene expression profiles between castes: queens show more than 2000 differentially expressed genes relative to reproductive or sterile workers, and even within the worker caste (reproductive workers vs sterile workers), there are more than 200 genes showing altered expression patterns [13].

One of the most extreme and intriguing systems of worker reproduction in honeybees is found in the Cape honeybee, *A. m. capensis,* in South Africa. In this subspecies, workers are able to lay unfertilized eggs, which are diploid due to a spindle rotation failure during meiosis, [61] giving rise to female offspring (thelytoky) [62]. Similarly to the anarchistic honeybees, workers of the Cape honeybee can also activate their ovaries in presence of the queen [63]. These workers are very queenlike in general, have a spermatheca, produce large quantities of queen-like MGP [64], and can produce parthenogenetically diploid female offspring. Using a backcross it was shown that the type of parthenogenesis is influenced by a single recessive gene [65], which was subsequently mapped to chromosome 13 [66]. The gene has been shown to have strong pleiotropic effects, which is reflected in the phenotypic associations

between the type of parthenogenesis, the amount of queen-like MGP, and the onset of egg-laying [66]. The strongest candidate gene within the mapped region is the transcription factor *gemini*, which is alternatively spliced with four highly abundant transcript variants [67]. Two exons are affected by alternative splicing, exon 5 and 7. Transcript variants differ between queens, reproductive workers, and sterile workers. Transcript variant specific knock-down using RNAi, resulted in rapid ovary activation in otherwise sterile workers. The difference in splicing may be due to a 9 bp intronic sequence, which has the typical sequence motif of a splice enhancer. Sterile altruistic lineages of honeybees possess this splice motif and thus produce transcript variants that establish sterility. By contrast, Cape honeybees lack this short motif, which might give rise to their unusual reproductive behavior.

Concluding remarks

Caste determination in honeybees is established by nutrient sensing pathways that translate environmental signals derived from the quality and quantity of food into different hormonal signaling via JH released from the *corpora allata*, ultimately triggering differential gene expression. Gene expression differences in the queen and worker castes are due to differences in *cis*-regulatory elements and DNA methylation patterns. However, it is unclear what evolutionary processes established these differences, and in particular why worker-specific genes are more strongly methylated. This may be related to the less effective molecular evolution of worker-specific genes, but a direct connection between these observations has not yet been made. To date, much of what we know about the genetics underlying social behavior is based on associations and correlations, and therefore rather descriptive. In the end however, a comprehensive understanding will require well controlled knock down experiments applying methods like RNAi or eventually transgenic honeybees with targeted knock out mutants to rigorously test for causal associations between gene expression and phenotype. A number of candidate genes influencing the complex social behavior of worker

bees have been identified through QTL mapping, candidate gene expression and transcriptome studies as well as experimentally induced knock-outs of gene expression by means of RNAi. Further advances into our understanding of the genetics and molecular mechanisms of social behavior are to be expected from genome-wide association and transcriptome studies utilizing independently evolved lineages that might also differ in certain major life history characteristics. The recent vast developments of "omics" technologies [68] will allow for integrative approaches, comparing a number of social insect species and greatly facilitating the identification and understanding of the networks, pathways, and genes that are ultimately responsible for different forms of social behavior.

Box 1. Biology of the Honeybee

Honeybee colonies are headed by a single queen that leaves the nest for nuptial flights 7-10 days post emergence. Mating takes place in flight at a drone congregation area (DCA) where thousands of drones gather for mating. A queen usually mates with 10-20 drones [69] and returns to the colony and activates its ovaries before initiating oviposition. Queen fecundity is exceptionally high, laying up 2000 eggs per day during the peak season.

Queens can lay unfertilized and fertilized eggs, the former developing into males whereas the latter give rise to females. This form of sex determination is based on the action of alleles at a single gene, the *complementary sex determiner* (*csd*) [70]. Individuals that are heterozygous at *csd* develop into females, whereas hemizygous (unfertilized eggs) and homozygous (fertilized eggs) will develop into males. Homozygous males represent a huge fitness cost at the colony level as they do not contribute to the work. Workers are able to detect diploid males and selectively remove diploid male larvae by cannibalizing them [71]. Thus, the *csd* gene is under strong negative frequency-dependent selection (rare alleles have a high fitness advantage). Fertilized eggs developing into female offspring will develop into queens or workers depending on the nutrition of the larvae (see *Reproductive Division of Labor - Caste Determination*). Due to the multiple mating of the queen, females belong to different subfamilies depending on the source of the sperm used to inseminate the egg. Within subfamilies, relatedness is high (r = 0.75) forced by the asymmetry due to the haplodiploid genetic system. However, between subfamily relatedness converges towards r = 0.25 with increasing numbers of mates of the queen assuming males are unrelated. As honeybee colonies are perennial, colonies hibernate as a unit with the queen accompanied by about 10,000 workers. In temperate regions workers form a winter cluster to protecting the colony from cold temperatures. Winter bees may survive for three months, whereas worker bees in

spring and summer show the typical age polyethism ending up as foragers typically live 4-6 weeks. Queens may live up to three years.

Box 2. The genome of the honeybee

The genome of the honeybee consists of 262 MB arranged in 16 chromosomes. The largest, chromosome 1, is metacentric, whereas the other 15 chromosomes are acrocentric. The recombinational length of the genome is 4114.5 cM resulting in a recombination rate of 15.7 cM/Mb [72], one of the highest amongst multicellular eukaryotes. The genome is characterized by a high A+T content (>70%) and lacks most of the major families of transposons. In comparison to other sequenced solitary insect genomes, the honeybees contain less genes of the innate immune system, detoxification enzymes, cuticle forming proteins, and gustatory receptors, whereas they contain more genes encoding odorant receptors. Novel genes were found for nectar and pollen utilization. The honeybee genome contains genes for a fully functional DNA methylation system [26]. The genome shows a dichotomy in the distribution of CpG sites [73], because hypomethylated genes are associated with developmental processes whereas genes that are hypermethylated in the germline are associated with fundamental biological processes. Genes with a caste-specific expression pattern predominantly belong to the high-CpG class (hpyomethylated) [73].

Besides the availability of the genome sequence several genomic and proteomic tools are available that might enhance further molecular studies of social behavior, including a microsatellite linkage map comprising 2000 markers [72], a 44K SNP array [74], a high-density oligo-nucleotide array [2], and an organ-level protein atlas [68]. Further genome sequencing projects are underway to improve the current *A. mellifera* assembly, as well as the

de novo sequencing of other honeybee species including *A. florea* (dwarf honeybee) and *A. dorsata* (giant honeybee).

Box 3. Major genetic study systems of *A. mellifera*

Genetic studies of honeybees have been hampered by two obstacles. First, all attempts to use targeted mutagenesis on honeybees including radiation, chemical mutagenesis or transposon-mediated mutagenesis have failed. Even if transgenic lines can be established, mutations need to end up the germ line. Assuming sperm storage becomes more efficient, in the end it is the queen bee which needs to carry the mutant. As queens cannot be kept in isolation, every mutant strain needs to be kept in a set of colonies, which becomes a major logistic problem for the maintenance of phenotypic mutants. The obvious difficulties in terms of infrastructure and overall costs have led to the disappearance of many of the mutant lines in the past decades from research institutions. Although attempts for generating transgenic bees are underway [75], in the past these challenges have been overcome by using special honeybee populations.

1) A bi-directionally selected population differing in the amount of pollen hoarding has been established using 400 colonies that were selected based on the colony phenotype. Selection was applied for several generations and intercrosses were used to establish mapping populations for QTL analyses. High- and low pollen hoarding strains are currently maintained at the Arizona State University in Tempe.

2) A rare colony phenotype was identified in a large screen searching for colonies with signs of worker reproduction, as indicated by brood nests occurring above the queen excluder, a beekeeping device that allows workers but not the queen to pass through a barrier based on their physical size. As those colonies showed worker reproduction in the presence of the queen they have been termed “anarchistic bees”. Initially, 9% of

the worker population showed activated ovaries, which increased to 40% through subsequent selection for the anarchistic phenotype [57].

3) The Cape honey bee, *Apis mellifera capensis* (Fig. 2), endemic to the Western Cape region of South Africa shows some peculiarities tightly linked to the workers ability to produce female (diploid) eggs parthenogenetically (thelytoky) as a result of aberrant meiosis. This results in nearly clonal offspring as chromosomal segregation is suppressed and crossing-over events are reduced. Selfish selection is predicted due to the ability to produce female offspring and indeed, workers are much more queen-like than workers of other subspecies. They have a spermatheca, large ovaries, queen-like mandibular secretions, and the ability to attract nurse workers who will to feed them with royal jelly. These features predispose Cape honey bees for a socially parasitic strategy. In the 1990's a few hundred hives of Cape bees were transported to the northern regions of South Africa for pollination services. Cape bee workers were transferred to colonies of the related subspecies *A. m. scutellata*, where upon they killed the resident queen and established themselves as reproductive parasites, exploiting the cooperative brood care of host workers. A single clone of the Cape bee did spread all over South Africa destroying thousands of colonies (the "capensis calamity").

Box 4. Reproductive Groundplan Hypothesis (RGPH)

The "reproductive groundplan hypothesis" (RGPH) was developed based on the results of phenotypic associations of reproductive characters and foraging preferences derived from the selection experiment for high- and low-pollen hoarding strains. It aims at explaining the evolution of queen and worker castes in social insect species through changes in the gene

regulatory network influencing and regulating foraging and reproductive cycles in the solitary ancestors of social insects [76,77]. The temporal shift of expression of maternal care genes has been adapted for sib-care performed by workers. The tight linkage of maternal behavior to the reproductive status opened the way for the evolution of a sterile worker caste [77]. This link also influences the division of labor within the worker caste as foraging for pollen is correlated to ovary size which is affected by high titers of the egg yolk protein *vitellogenin* [78].

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Glossary

polyphenism: different (discrete) phenotypes arise from a single genotype by exposure to different environmental conditions.

bi-directional selection: selection towards the two extremes of a certain phenotype.

instar larvae (first, second, third etc.): developmental stages of insects separated by molts.

intercaste: individuals that show an intermediate phenotype in species that show otherwise a distinct polyphenism within the female sex (queens and workers).

emergency queen rearing: in honeybees workers rear a new queen, if the resident queens disappears (due to death, experimental removal etc.) using available young larvae (1^{st} and 2^{nd} instar larvae).

corpora allata (pl.): pair of endocrine gland attached to the brain of insects that produces juvenile hormone, which is secreted directly into the hemolymph.

polyethism: occurrence of different worker behaviours within a social insect colony. Temporal polyethism refers to changes in these behavior as individuals age.

thelytoky: type of parthenogenesis, which results in female-only offspring. In order to ensure diploidy of offspring thelytokous parthenogenesis, either meiosis is suppressed (apomictic) or haploid meiotic products fuse (automictic).

Figure 1.

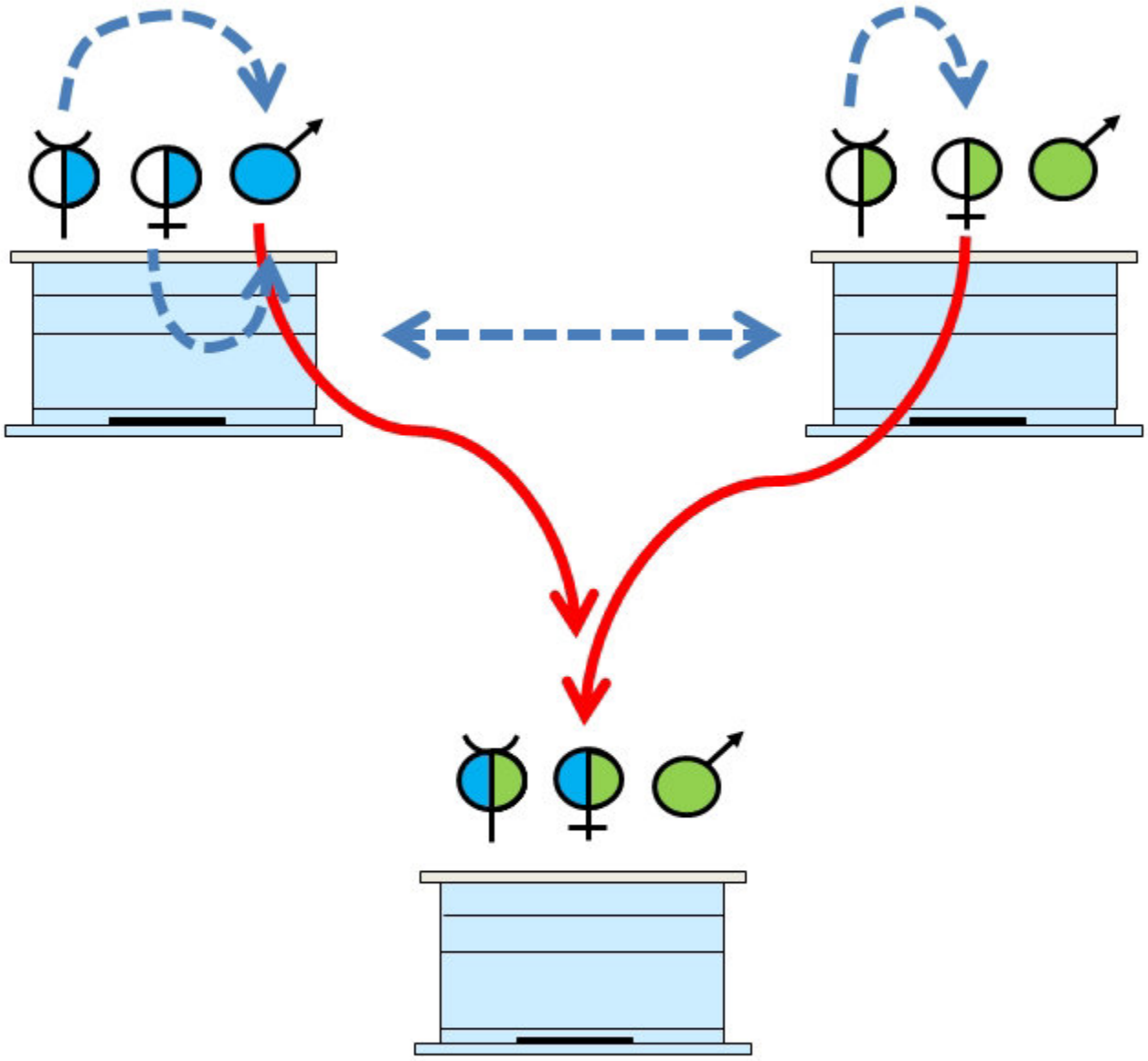
Direct and indirect transmission routes of genes in honeybees. Red lines indicate direct transmission by queens (right side) or drones (left side). Genes specific for these individuals are exposed directly to selection. Blue lines (dashed) show indirect transmission routes, which are the main route for workers genes, which might be transmitted by queens (right side) or drones (left side). Worker exclusive genes are exposed to selection always in the other sex (drones) or the other caste (queens). Direct transmission of worker genes via worker-laid drones is negligible, as only 0.1% of all males are worker produced [79]. An additional level of selection is between colony selection, which is affecting all genes of all individuals indirectly.

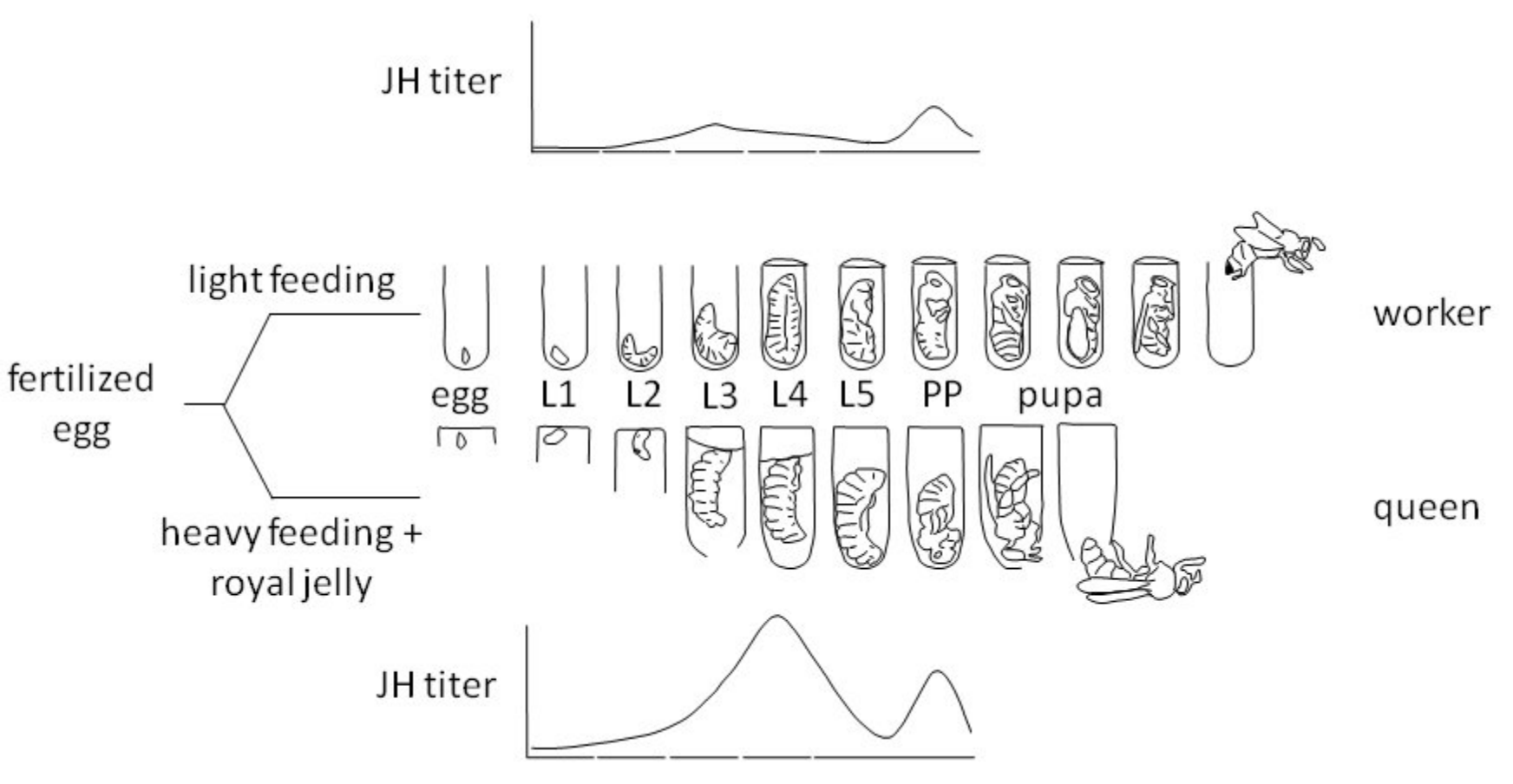
Figure 2.

Queen and worker caste of the honeybee (*Apis mellifera*). **A.** Cape honeybee worker (in the center, with brownish abdomen) induces a retinue of workers of *A. m. scutellata*. Cape honeybee workers produce large quantities of the queen pheromone 9-ODA. Photo by S. Härtel. **B.** Developmental dichotomy of female larvae in the honeybee. The upper panel shows the development of workers including their juvenile hormone (JH) titer during different phases of development. The lower panel shows the development of queens including their JH titers. Modified after [12] and [18].

Table 1. QTL studies in honeybees related to social behavior.

Behavioral category	**Trait**	**Number of QTLs**	**Effect size [% phenotypic variance]**	**# of candidate genes**	**Refs**
Defensive behavior	Stinging behavior	7	n.d.	n.d.	[35]
	Guarding behavior	7	n.d.	n.d.	[80,81]
	Venom components (five compounds)	9	20-42	1-43	[82]
	Alarm pheromone levels (four compounds)	7	40 (for n-decyl-acetate) n.d. (other QTL)	n.d.	[83]
Worker reproduction	Worker sterility	4	5-8	86 (QTL1) n.d. (QTL2-4)	[58]
	Ovary size	5	n.d. 14-18	53-135 84-107	[84,85]
	Ovary asymmetry	1	4-29	83	[85,86]
Foraging	Foraging behavior; Age at first flight; Sucrose responsiveness	4	38 (*pln1*) 33 (*pln2*) 10 (*pln3*) n.d.	1 (each QTL)	[44,45,87,88]
learning	Reversal learning	2	13-14	n.d.	[89]
	Latent inhibition	1	28	n.d.	[89]




JH titer
light feeding
fertilized egg
heavy feeding + royal jelly
egg
L1
L2
L3
L4
L5
PP
pupa
worker
queen
JH titer