11 Endocrine Control of Insect Polyphenism

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Summary

Polyphenism in insects is associated with some of the most striking and successful life histories. Insect polyphenisms center around four major themes: wing length variation (including winglessness), differences in fertility or reproductive strategies, body and/or wing coloration and exaggerated morphologies. Hormones, especially juvenile hormone and ecdysteroids are important factors underlying the generation of distinct morphologies and reproductive strategies, and the insulin-signaling pathway is now also emerging as a major player. We present an overview of current knowledge regarding wing length dimorphism in crickets, the gradual phase shift from the solitary to gregarious syndrome in migrating locusts, the complex switching between wingless/winged forms and asexual/sexual reproduction in aphids, wing and body color variants in butterflies, male horn dimorphism in beetles, and caste polyphenism occurring in the hemimetabolous termites and holometabolous hymenopterans.

11.1. Polyphenism and Polymorphism: Discontinuity in the Variation of Insects

Historically, the terms polymorphism and polyphenism have the same meaning, namely intraspecific variation in sets of characters. This phenomenon is seen as an adaptive response that allows genotypes to track short-term cycles of environmental variation and, at the same time, maintain cohesiveness of adapted gene complexes. With recent advances in population genetics, enzymology, genomics, and proteomics the term polymorphism has now gained a much wider meaning. It has come to denote variation in nucleotide sequences in general. This genetic variation may or may not have consequences for phenotypic character states, depending on whether it occurs in coding regions, promoter and regulatory regions, or in selectively neutral DNA, such as microsatellites.

In this chapter we use the term polyphenism to describe, in a more narrow sense, the occurrence of intraspecific variation in phenotypes. Specifically, we focus on relatively discrete or discontinuous variation in phenotype expression. Polyphenic mechanisms can be described at two levels: (1) cues that serve as proximate triggers of developmental alternatives and (2) endogenous response cascades that drive the developmental responses. Triggering stimuli may consist of external environmental factors (e.g., photoperiod, crowding), internal genetic factors (e.g., alleles at polymorphic loci), or a mixture of the two. Endogenous response cascades involve a series of systems that relay, synchronize, and coordinate differentiated processes in target systems.

The most common form of polyphenism in animals is sexual dimorphism. This, in most cases, is triggered by genetic factors that generate distinct phenotypes that then act at the tissue level through a series of endocrine response cascades. Even though sexual polyphenism occurs to some degree in all insects, we consider it only peripherally in this chapter. Rather, and following the tradition already set forward in the review on this subject

DOI: 10.1016/B978-0-12-384749-2.10011-1

by Hardie and Lees (1985), and building on our prior version of this review (Hartfelder and Emlen, 2005), we focus on distinct phenotypes (in either or both sexes) that are triggered by environmental or other exogenous factors. When reviewing recent studies on this subject and comparing these to insights already presented in the 2005 version of this chapter, it became clear that considerable progress has been made in the elucidation of endogenous response cascades underlying polyphenic trait expression in some insect polyphenisms, and these now truly represent model systems. Major progress in the field can be attributed to genomic resources and high throughput platforms for both transcriptome and proteome analyses. These were championed for the honey bee, which became the first polyphenic (social) insect to have its genome sequenced (The Honey Bee Genome Sequencing Consortium, 2006). Next came the genome of the pea aphid *Acyrthosiphon pisum,* which has recently been concluded and published (International Aphid Genomics, 2010) and the road has been paved for several other genomes.

11.2. Polyphenism in the Hemimetabola

Wing length is the most predominant form of polyphenism in hemimetabolous insects. It involves differential investment in wings and wing muscles, and appears to reflect an ecophysiological trade-off in resource allocation between dispersal and reproduction. It is observed in species that colonize and/or exploit ephemeral resources, and may be integrated into life histories of considerable complexity, such as seen in locust and aphid phase polyphenism and in termite caste polyphenism. In the following sections, we will review the current status of endocrine regulation of wing, phase, and caste polyphenism encountered in the Hemimetabola.

11.2.1. Hemiptera/Homoptera Wing Polyphenism

11.2.1.1. Soapberry bugs A number of hemipteran species have polyphenic wing expression (Dingle and Winchell, 1997; Tanaka and Wolda, 1987). The hemipteran *Jadera haemotoloma* (the soapberry bug) exhibits a wing polymorphism as four morphs: three winged forms, one that always has viable flight muscle, one that histolyzes flight muscles part way through the adult stage, and a winged form that never produces viable flight musculature, as well as a completely wingless form that also has inviable flight musculature (Dingle and Winchell, 1997). *Jadera haemotoloma* has undergone rapid recent evolution following a host-shift that occurred within the past 50 years (Carroll and Boyd, 1992; Carroll *et al.*, 1997, 2001), and one characteristic that has changed dramatically is the proportion of the population expressing wings: derived populations (on the new host) have significantly fewer winged animals than ancestral populations.

Dingle and Winchell (1997) demonstrated that this polyphenism is regulated by a threshold mechanism, and that this threshold behaves like a polygenic character with high levels of additive genetic variance. Manipulations of the amount of nymphal food predictably affected the percent of winged offspring, as did topical applications of methoprene (Dingle and Winchell, 1997). In laboratory animals sampled from an ancestral population, increases in the amount of food decreased the percent developing with wings. Similarly, experimental augmentation of juvenile hormone (JH) levels at the beginning of the final nymphal instar also decreased the proportion of winged animals, suggesting that high levels of JH result in a reduction of the relative amount of wing growth (Dingle and Winchell, 1997).

There is no information regarding the sensitivity of animals to JH during the penultimate or earlier instars, nor of the relative levels of ecdysteroids. Nevertheless, results available at this time show striking similarities with the polyphenic mechanism described for crickets (Section 11.2.2.1.). The timing of the sensitive period, the implicated role for JH, and the direction of the effect of JH all agree with the basic model for cricket wing polyphenism.

11.2.1.2. Planthoppers Planthoppers often occur in both winged and wingless forms (Denno and Perfect, 1994; Kisimoto, 1965; Morooka *et al.*, 1988), and dimorphism in these species results from a threshold mechanism with heritable variation for the threshold (Denno *et al.*, 1986, 1996; Matsumara, 1996; Morooka and Tojo, 1992; Peterson and Denno, 1997). Where studied, the environmental factor most relevant to wing expression appears to be population density, or crowding, and as with the wing polyphenism already described, the physiological response to nymphal crowding appears to be mediated by levels of JH.

To date, the endocrine regulation of wing polyphenism has been best studied in the brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae). The default developmental pathway appears to involve wing production, but Iwanaga and Tojo (1986) showed that both exposure to solitary (low density) conditions and topical applications of JH could suppress wing development and result in a greater proportion of wingless individuals. They identified sensitive periods in the prepenultimate (third) and penultimate (fourth) instars (Iwanaga and Tojo, 1986). The timing of these sensitive periods and the effect of JH were corroborated by Ayoade *et al.* (1999), who induced wingless morphs in a strain of planthoppers that had been selected to be entirely winged.

Bertuso *et al.* (2002) then induced expression of wings in a line selected to be entirely wingless by applying precocene to animals at these same sensitive periods. Dai *et al.* (2001) measured JH titers in presumptive winged and wingless nymphs and found that putative short-winged individuals had higher JH levels and lower juvenile hormone esterase (JHE) levels than putative long-winged individuals. Liu *et al.* (2008) showed that these patterns re-emerge in the fifth instar, with nymphs developing into short-winged adults having higher JH titers and lower JHE levels from 48 h onward during the fifth instar.

The two morphs also showed divergent titer levels in the adult stage, where a higher JH titer in the short-winged morph was associated with an earlier development of oocytes (Bertuso *et al.*, 2002), thus providing evidence for the dual (developmental and reproductive) role of JH in the divergent life history strategies of planthoppers. Tufail *et al.* (2010) measured vitellogenin (Vg) profiles and showed that levels increase earlier in short-winged adults than in long-winged adults (on days 3 and 4, respectively), and that topical application of JH could induce upregulation of vitellogenin transcription. The delayed rise in Vg exhibited by long-winged females is consistent with the hypothesis that this morph is capable of long-distance flights prior to reproduction.

Thus, wing polyphenism in planthoppers, as with wing polyphenisms in soapberry bugs and crickets, appears to be regulated by JH during brief sensitive periods late in nymphal life. Genomic resources are now available for the brown planthopper (Noda *et al.*, 2008), and this has permitted researchers to characterize the JHE gene (Liu *et al.*, 2008) and the farnesoic acid O-methyltransferase gene (an enzyme critical for JH biosynthesis; Liu *et al.*, 2008), as well as the vitellogenin gene (Tufail *et al.*, 2010), and studies on differential gene transcription between winged and wingless morphs are likely to be forthcoming.

11.2.1.3. Aphid phase and caste polyphenism Aphids are a monophyletic group represented by around 4400 species worldwide which, in terms of life cycle complexity, are outstanding champions. Aphid life cycles can include at least two different forms of polyphenism, (1) cyclical switching between asexual reproduction (viviparous parthenogenesis) and sexual reproduction (associated with the production of haploid eggs capable of overwintering after fertilization), and (2) switching between a wingless sedentary morphology, and a winged morph capable of dispersal. This switching capacity enables aphids to successfully colonize and thrive on ephemeral habitats. In some cases, these polyphenic switches occur simultaneously (e.g., the switch from asexual to sexual reproduction may coincide with a switch from wingless to winged morphologies), or they may occur separately (the switch from wingless to winged morphologies can occur without coincident changes in mode of reproduction or host plant preference). The large variation in species-specific aphid life cycles stems from the fact that these elements of polyphenism can be shuffled and integrated as seemingly independent modules in the evolutionary history of each species.

In this chapter we focus on the ontogenetic mechanisms that generate aphid polyphenism (for reviews of aphid life cycles and their ecological significance see Braendle *et al.*, 2006; Dixon, 1977, 1998; Moran, 1992). In an effort to facilitate the discussion of aphid polyphenism, and to place these mechanisms within the context of insect polyphenism in general, we present a simplified description of aphid life cycles and adapt a simplified terminology (Blackman, 1994).

11.2.1.3.1. Asexual versus sexual reproduction All present day aphids reproduce parthenogenetically, a capacity which appears to have been acquired from a common ancestor approximately 250 million years ago. Yet, only a small number of derived species (i.e., those at the tips of the different branches in the phylogenetic tree) are strictly parthenogenetic (Simon *et al.*, 2002). Instead, the majority of aphid species facultatively switch between parthenogenetic and sexual modes of reproduction.

During the asexual phase, females give birth to parthenogenetic progeny that develop completely within the ovaries (for detailed reviews on the embryology of sexually versus asexually developing offspring see Le Trionnaire *et al.*, 2008; Miura *et al.*, 2003). While these progeny develop, their own ovaries become active and these embryos begin ovulation before being born. In this extreme telescoping of generations, both daughters and granddaughters develop simultaneously within a single adult female. This parthenogenetic/paedogenetic reproductive strategy of wingless female aphids is highly efficient for colonizing ephemeral habitats, because it can very rapidly generate large numbers of progeny. However, rapid production of parthenogenetic daughters can also lead to overcrowding, and with this, the need to disperse to new host plants (see Section 11.2.1.3.2.).

At the end of a parthenogenetic phase of reproduction, and generally at the end of the favorable season, female aphids begin producing sexually reproductive offspring. Often, this transition from asexual to sexual reproduction is coupled with a shift from the primary to a secondary host plant species. Aphids that live on a primary host plant with a short seasonal duration may switch to a secondary host plant, and this can occur in two ways, depending on the species. Asexual females may produce a generation of winged daughters that first disperse to the new host plant and then produce sexually reproductive (male and female) offspring. Alternatively, asexual females may produce winged males and females directly and these then disperse to the secondary host and reproduce sexually. In either case, the cycle is complete when daughters

disperse back to the primary host the following season and begin parthenogenetic reproduction anew.

As may be expected, given the strict seasonality in both host plant alternation and cyclical parthenogenesis, photoperiod appears to be an important environmental cue for this polyphenism. The effects of photoperiod on these key elements in aphid life cycles have long been recognized (reviewed in Hardie and Lees, 1985), and have been studied extensively, especially in the black bean aphid, *Aphis fabae*, and the vetch aphid, *Megoura vicinae* (Hardie, 1981b,c; Hardie and Lees, 1983). The photoperiodic response in the switch from parthenogenetic to sexual forms depends primarily on the length of the scotophase (Hardie *et al.*, 1990), and immunocytochemical analyses of rhodopsins and phototransduction proteins suggest that photoperiodic receptors are located in the anterior dorsal region of the aphid protocerebrum (Hardie and Nunes, 2001).

Once the external trigger (changes in photoperiod, especially scotophase length, and to a lesser extent temperature) has been detected, the polyphenic switch between modes of reproduction is coordinated by circulating levels of hormones, in particular by JH. Specifically, in *A. fabae,* topical applications of JH inhibited the production of haploid eggs (and hence the switch from asexual to sexual reproduction), and promoted parthenogenetic development (Hardie, 1981b,c). JH also affected host plant preference behavior at this same stage (Hardie, 1980, 1981a; Hardie *et al.*, 1990), which makes sense, since these events occur simultaneously in *A. fabae*, and since both appear to involve a response to photoperiod. Based on these results, Hardie and Lees (1985) proposed that the endogenous JH titer should be high during long-day conditions, when animals feed on the primary host plant and reproduce parthenogenetically, and low later in the season when aphids switch from asexual to sexual reproduction and from primary to secondary host plants.

11.2.1.3.2. Wingless versus winged morphology The switch from wingless to winged morphologies can occur in two different situations. First, parthenogenetically reproducing aphids overcrowd their primary host plant and must disperse to other (also primary) host individuals. As conditions become crowded, females begin producing asexual daughters with wings. These winged females disperse to new plants and begin parthenogenetic production of wingless daughters all over again.

At the end of the favorable growing season, when conditions begin to deteriorate everywhere (e.g., as the primary host plants begin to die), aphids again produce a winged generation of offspring, except that this time the switch between wingless and winged morphologies may coincide with both the switch from primary to secondary host plants, and also with the switch from asexual to sexual reproduction. Thus, the first manifestation of the wingless versus winged switch is not associated with a simultaneous switch in the mode of reproduction, but the second one is. This suggests that at least partially independent mechanisms may be involved. In some species the winged forms produced at these two times are morphologically distinct (Hille Ris Lambers, 1966), which is consistent with the idea that separate environmental cues and/or endocrine mechanisms may regulate wing expression at these two stages.

Early research on the mechanism of aphid wing polyphenism produced equivocal results, probably due to difficulties distinguishing between hormonally induced "juvenilization" of animals and polyphenism-specific effects on wing expression per se (reviewed in Hardie and Lees, 1985). The complex life cycles of aphids and the simultaneous involvement of several different forms of polyphenism made this problem still more difficult. However, several clever experiments with *A. fabae* finally resolved this dilemma and clearly established a role for JH in one form of aphid wing polyphenism (Hardie, 1980, 1981b,c; Lees, 1977, 1980). Juvenile hormone appears to regulate the switch from wingless to winged morphologies that occurs at the end of the growing season (and coincident with the switch from asexual to sexual reproduction). Animals harvested at a stage that would typically begin production of winged offspring (i.e., at the end of the season) could, if exposed to either long days or high temperatures (i.e., summer conditions), be induced to forego wing production and produce wingless daughters instead. This effect of environmental stimuli could be mimicked by topical applications of JH I at this same time, suggesting that high levels of JH are associated with continued production of wingless offspring, and that a seasonal decline in levels of JH may underlie the polyphenic switch from wingless to winged morphologies (as it did with the end-of-season switch between asexual and sexual reproduction).

In contrast, the most important environmental trigger of wing production in conditions of crowding is an increase in tactile stimulation between individuals (Johnson, 1965) or a response to the release of aphid alarm pheromone due to predator pressure (Hatano *et al.*, 2010; Kunert and Weisser, 2005). Thus, animals reared under crowded conditions produce winged progeny irrespective of photoperiod or temperature and even when exposed to topical applications of JH (Hardie, 1980). These results suggest that the two forms of wing polyphenism are regulated by independent endocrine mechanisms. In particular, they suggest that polyphenic production of winged morphologies during the period of parthenogenetic reproduction occurs by a mechanism other than circulating levels of JH, and one that can operate independent of levels of JH (i.e., by a mechanism capable of inducing wing expression even when environmental conditions stimulate high levels of JH).

Major obstacles to direct assessment of JH titers and corpora allata (CA) activity in aphids are their small size and telescoping of generations. Chemical allatectomy by precocenes turned out to be a valuable research tool in this context, even though not all aphid species were equally susceptible (Hardie, 1986), and not all precocene compounds acted in the same direction (Hardie *et al.*, 1995, 1996). Juvenile hormone rescue experiments on precocene-treated larvae of a pink *A. pisum* clone (Gao and Hardie, 1996) showed that the destruction of the CA by precocene II or precocene III resulted in precocious adult development. The alate-promoting property of these compounds appeared to be unrelated to the decreased JH titer, and instead depended heavily on population density (Hardie *et al.*, 1995). In contrast, flight muscle breakdown in alate adult *A. pisum* that had undertaken a migratory flight to a new host plant has been shown to be under JH control (Kobayashi and Ishikawa, 1993, 1994). Thus, there is substantial evidence for the role of JH in specific contexts of developmental regulation in aphids, but it is clearly not the general role that was attributed to it in the early days of aphid endocrinology. A role for JH in wing polyphenism has also been called into question by results of direct JH III titer determination using an LC-MS approach on pools of *A. pisum* aphids preferentially producing winged or unwinged offspring. As shown by Schwartzberg *et al.* (2008), there was no evidence for differences in JH titer between the two groups.

To our knowledge, little information exists on the role of ecdysteroids in wing phenotype expression of aphids, yet genomic resources have now enabled the annotation of genes involved in ecdysteroid biosynthesis as well as nuclear receptors, and modeled the possible binding properties of 20-hydroxyecdysone (20E) to a putative ecdysone receptor (Christiaens *et al.*, 2010). While there is little doubt that ecdysteroids play a role in the regulation of molting and metamorphosis in general, in aphids their role in wing polyphenism and/or cyclical parthenogenesis still warrants functional validation.

The proportion of winged females produced in response to a given environmental cue may vary between clonal genotypes, indicating genotype–environment interaction on this important trait of adaptive plasticity (Braendle *et al.*, 2005a,b). An even stronger influence of genotype has been detected in males of *A. pisum,* where wing production is under the control of a single locus on the X chromosome (Cauillaud *et al.*, 2002). Allelic variation in this locus, *aphicarus (api),* is associated with clonal genotype differences in the propensity to produce winged males (Braendle *et al.*, 2005b). This finding is in line with earlier experiments that showed that precocene-mediated allatectomy was much more effective in apterizing presumptive alate females than males in this species (Christiansen-Weniger and Hardie, 2000).

Interestingly, apterization of presumptive alates can also occur as a consequence of parasitization during the early larval instars (Johnson, 1959). Parasitization effects on wing development appear to be regulated independently of metamorphosis, and in particular, seem to occur independent of JH (Hardie and Lees, 1985). In pea aphids, parasitization by *Aphidius pisum* caused apterization and other correlated changes in body shape (Christiansen-Weniger and Hardie, 2000), yet parasitization of the same species by *Aphidius ervi* had the opposite effect, resulting in a higher proportion of winged offspring (Sloggett and Weisser, 2002). The developmental mechanisms underlying the divergent responses to parasitization are not yet understood.

In conclusion, the mechanisms underlying aphid phase polyphenism are responses contingent on different environmental cues and may involve completely different endocrine mechanisms. Thus, wing polyphenism represents not one, but two developmental mechanisms. Again, superficially taken, reproductive polyphenism would appear to be very different from wing polyphenism, yet it occurs at the same time as one of the wing polyphenisms in response to the same environmental stimulus, and is regulated in the same direction by the same hormone (JH) (**Figure 1**). Thus, a useful description of aphid polyphenism may involve a classification scheme that distinguishes an "autumn" polyphenism that includes both wing morphology and mode of reproduction, and which is regulated by a seasonal change in photoperiod and a corresponding decline in levels of JH, from a "summer" polyphenism. This seasonal change only involves wing expression, which occurs in response to crowding and incorporates an as yet unidentified endocrine mechanism, independent from circulating levels of JH.

11.2.1.3.3. Soldier aphids It is worth noting that aphids, the masters of phase polyphenism, exhibit yet another form of polyphenism. Some aphid species facultatively produce a behaviorally and morphologically distinct soldier caste. Since its original description (Aoki, 1977), this phenomenon has been detected in over 50 aphid species in the families Pemphigidae and Hormaphididae (Stern *et al.*, 1997). Most studies of aphid soldiers have focused on clonal relatedness and kin selection (Abbot *et al.*, 2001; Carlin *et al.*, 1994; Pike and Foster, 2008), as well as on colony division of labor and defense against predators (Foster and Rhoden, 1998; Fukatsu *et al.*, 2005; Rhoden and Foster, 2002; Schutze and Maschwitz, 1991). Little is known about proximate mechanisms that induce soldier formation. Positive correlations have been found between soldier proportion and colony size (Ito *et al.*, 1995), which may be mediated through direct contact stimuli with non-soldier aphids in a colony and negative feedback from contact with soldier aphids (Shibao *et al.*, 2010). Ontogenetically,

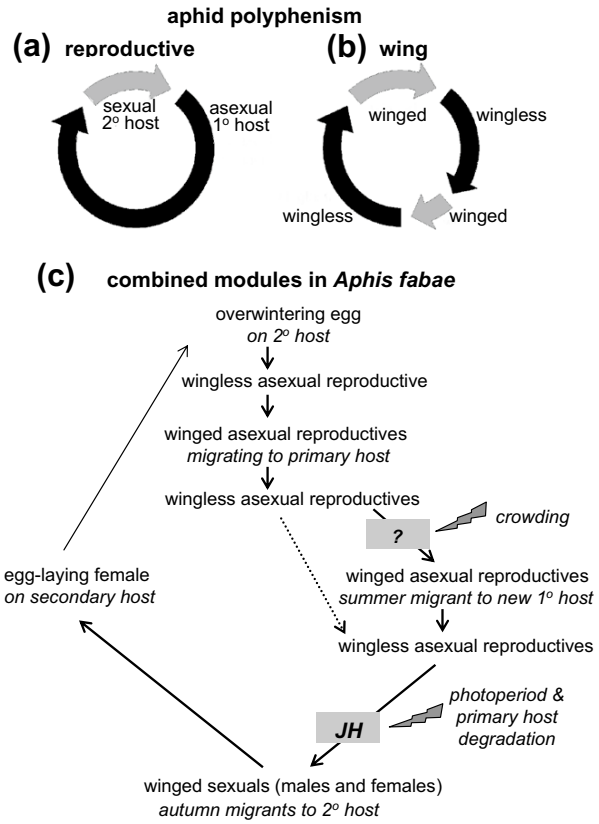

Figure 1 Polyphenism in aphid life cycles can tentatively be assigned to two separate modules. (a) Module 1 (reproductive polyphenism) represents the switch from asexual (parthenogenetic) to sexual reproduction. This switch may be associated with a switch from a primary to a secondary host plant. (b) Module 2 (wing polyphenism) represents the switch from a wingless to a winged form. (c) These modules appear combined in complex life cycles such as that of the bean aphid, *Aphis fabae*. In the latter, three switches from wingless to winged can occur in the annual cycle. The first one occurs when wingless (parthenogenetic) females that arose from overwintered eggs produce winged (parthenogenetic) daughters that migrate to the primary host. The mechanisms underlying this switch are little understood. In a rapid sequence of generations, a large population of wingless (parthenogenetic) females then builds up on the primary host plant. When crowding reaches critical levels, some winged (parthenogenetic) females are produced. These colonize new primary host plants and initiate a new cycle of wingless generations. This switch from a wingless to a winged morph does not involve a switch in the mode of reproduction and appears to be independent of circulating (high) levels of JH. The third switch from a wingless to a winged morph occurs at the end of the favorable season when primary host plants degrade. This switch in wing expression now also involves a switch in reproductive mode, as the wingless asexual females start to produce winged males and females that mate and move to a secondary host plant. These either produce overwintering eggs themselves or produce a generation of egg-laying females. This third switch in wing expression is controlled by photoperiod (scotophase length) accompanied by a decrease in JH titers. Graph compiled from data by *Hardie and Lees* (1985) and *Hardie et al.* (1990, 1996).

the development of a soldier morphology appears to be linked to the replacement of the reproductive system by fat body cells and the lack of endosymbionts (Fukatsu and Ishikawa, 1992). Developmental trajectories have been mapped for *Pseuroregma bambucicola* (Ijichi *et al.*, 2004) and *Tuberaphis styraci* (Shibao *et al.*, 2010), inferring proximate cues that already act during embryonic stages and gradually separating developmental pathways in first instar nymphs.

11.2.1.3.4. Gene expression analyses and genomic resources A large collection of ESTs published in recent years (Ramsey *et al.*, 2007; Sabater-Muñoz *et al.*, 2006) and the establishment and use of microarray platforms (Brisson *et al.*, 2007; Le Trionnaire *et al.*, 2007; Wilson *et al.*, 2006) contributed to the establishment of a database of aphid genomic resources (Gauthier *et al.*, 2007). These efforts furthered the formation of a consortium for the sequencing and annotation of the first aphid genome, that of the pea aphid *A. pisum* (International Aphid Genomics Consortium, 2010). Furthermore, RNAi protocols have been devised for functional analyses of candidate genes or gene sets revealed by non-biased high throughput analyses (Jaubert-Possamai *et al.*, 2007; Mutti *et al.*, 2006).

Three noteworthy results from these novel approaches to aphid biology are summarized here, each hinting at significant insights to come. The first one concerns the perception of the photoperiod signal, which has long been attributed to an extraocular portion of the brain located within the region lateral to Group I neurosecretory cells (Steel and Lees, 1977). A transcriptome analysis of aphid heads revealed expression differences in cuticle protein encoding genes, suggesting a role in softening of the cuticle that overlies the photoreceptive area in short-day reared aphids (Le Trionnaire *et al.*, 2007). This approach also revealed alterations in the dopamine pathway (Gallot *et al.*, 2010; Le Trionnaire *et al.*, 2009), which may link both cuticle sclerotization/melanization in the head with neurotransmission, thus establishing a possible route for transgenerational signal transmission to the ovary.

A second important point is the annotation of a complete complement of DNA methyltransferases (Walsh *et al.*, 2010), bringing into focus the possible role of epigenetic alteration in the development of alternative phenotypes similar to findings in the honey bee (Kucharski *et al.*, 2008). The third point refers to the wing developmental gene regulatory network. Based on the *Drosophila* regulatory network, eleven genes were annotated in the aphid genome, and when assayed, six showed stage-specific variation and one gene, *apterous 1*, exhibited a significant difference in transcript levels between winged and unwinged morphs of *A. pisum* (for review see Brisson *et al.*, 2010) indicating that this gene may play a major role in polyphenic development.

11.2.2. Orthoptera

11.2.2.1. Wing polyphenism in crickets Facultative dispersal strategies in crickets involve the relative amounts of growth of the wings and associated wing musculature. Animals can have full-sized, functional wings (macropters), miniature wings (micropters), or be entirely wingless (brachypters). Wing expression and associated flight capability may differ among species (Harrison, 1980), among populations of a single species (Harrison, 1979), or even among time periods within a single individual's lifetime, with some crickets shedding their wings and histolyzing flight muscles after mating (Srihari *et al.*, 1975).

In a number of cricket species, wing expression is facultative and depends on environmental conditions encountered during nymphal development, such as temperature (Ghouri and McFarlane, 1958; McFarlane, 1962), photoperiod (Alexander, 1968; Masaki and Oyama, 1963; Mathad and McFarlane, 1968; Saeki, 1966a; Tanaka *et al.*, 1976), diet (McFarlane, 1962), and population density (Fuzeau-Braesch, 1961; Saeki, 1966b; Zera and Denno, 1997; Zera and Tiebel, 1988). In these taxa, wing expression appears to be regulated by a threshold mechanism and population comparisons, controlled-breeding and artificial selection studies all inferred considerable levels of genetic variation for the threshold of this polyphenism (Fairbairn and Yadlowski, 1997; Harrison, 1979; Roff, 1986, 1990; Zera *et al.*, 2007).

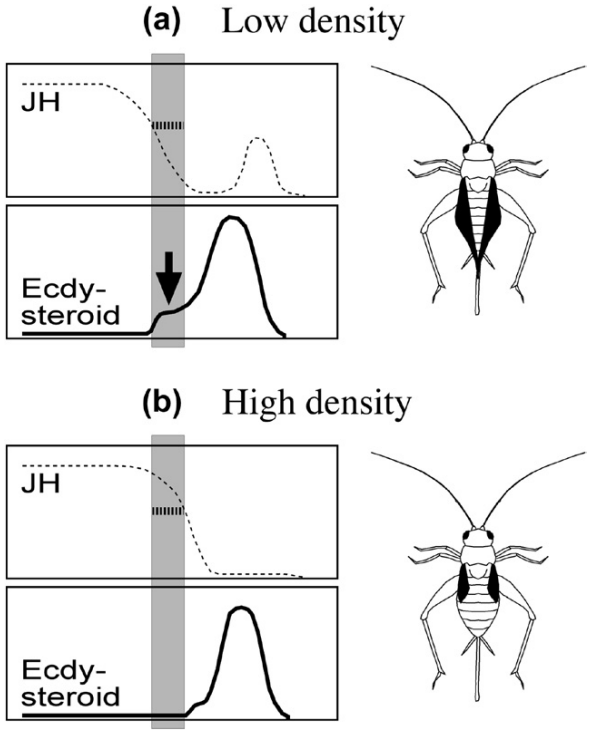

Figure 2 Facultative wing-length polyphenism in the cricket *Gryllus rubens* is a response to environmental conditions, especially nymphal crowding, and is controlled by a hormonal threshold mechanism acting during critical periods (shaded bars) in the two final instars of post-embryonic development. At low population densities, the JH titer drops below a critical threshold level due to enhanced JHE activity, whereas the pre-molting ecdysteroid titer exceeds a threshold level. This endocrine situation permits wing development to a macropterous phenotype. In contrast, high population densities lead to an above-threshold JH titer and a low ecdysteroid titer during this critical period. Consequently, wing development is inhibited and brachypterous adults are being formed. Graph compiled from data and models by *Zera and Tiebel* (1988), *Zera and Holtmeier* (1992), and *Zera and Denno* (1997).

The physiology of wing polyphenism has been most thoroughly studied in *Gryllus rubens* and *G. firmus*. Wing expression in these species is sensitive to population density, in particular, the level of crowding experienced by nymphs as they develop (Zera and Tiebel, 1988). Experiments that transferred animals between experimentally staged high and low densities revealed two sensitive periods relevant to expression of wings, during the middle of the penultimate and in the final nymphal instar, respectively (Zera and Tiebel, 1988). The default developmental pattern appears to be winged, but animals could be switched to a wingless fate if they were exposed to crowded conditions or received an exogenous JH application (Zera and Tiebel, 1988). This suggested that JH titer differences might underlie wing polyphenism, and this hypothesis was corroborated by direct measurement of JH titers in presumptive winged and wingless animals (Zera *et al.*, 1989). In addition, a second titer difference was observed. Animals destined to produce wings had higher levels of ecdysteroids than animals destined not to produce wings (Zera *et al.*, 1989).

Surprisingly, when comparing rates of JH biosynthesis in presumptive winged and wingless animals, Zera and Tobe (1990) found no differences, suggesting that morph-specific variation in levels of JH result from differential clearance of JH from the hemolymph, rather than from differential rates of hormone biosynthesis. This was ascribed to the enzyme JHE, which was shown to attain higher levels in winged than in wingless juveniles (Zera and Tiebel, 1989). Furthermore, the timing of these morph-specific differences in levels of JHE coincided with the two already described sensitive periods and with the observed morph-specific differences in the JH titer. A JHE has been cloned and sequenced in *G. assimilis* (Crone *et al.*, 2007) revealing a 19 bp indel in an intron that was strongly associated with differences in enzyme activity among lines selected for increased or decreased wing expression. This indicates that genetic differences resulting in different JHE function do not reside in the coding sequence but in possibly regulatory intronic motifs, a finding in accordance with previous findings that showed JHEs of short- and long-winged morphs do not differ biochemically (Zera *et al.*, 2002; Zera and Zeisset, 1996).

Based on these results, the following model was proposed (Zera and Denno, 1997; Zera and Holtmeier, 1992). Crickets have two sensitive periods, one during the middle of the penultimate and a second in the final nymphal instars. During these periods, wing development is sensitive to external environmental factors (crowding), and these external stimuli appear to result in morph-specific differences in levels of two hormones, JH and ecdysone. Animals destined to produce wings have lower levels of JH, and higher levels of ecdysteroids, than animals destined to mature without wings (**Figure 2**). Morph-specific differences in levels of JH appear to result from subtle differences in the timing of the decline in JH titers during these sensitive periods, and these differences in JH titers result from prospective winged animals having higher levels of the degratory enzyme JHE than prospective wingless ones. Thus, the default pattern of development entails production of wings, but a subset of individuals (animals with levels of JH above a genetically mediated threshold level) experience an early rise in levels of ecdysteroids, and this pulse of ecdysteroids probably "re-programs" these individuals toward production of a wingless body form.

While developmentally established during the nymphal phase, the density-dependent wing polyphenism has its major implications in the reproductive physiology of the adults. In a fusion of resource allocation and metabolic biochemistry studies, Zera and Zhao (2003) and Zhao and Zera (2002) demonstrated a genetic bias for flight-fuel synthesis (mainly triglycerides) in the flight-capable genotype versus a bias for ovarian lipids (phospholipids) in the flightless one. When JH was topically applied, the adult flight-capable morph shifted its lipid metabolism toward that of the flightless morph. In addition to this expected metabolic biphenism a surprising difference between the short- and long-winged adults emerged when JH synthesis and JH titers were measured. The long-winged adults exhibited a strong diurnal modulation in these parameters in the photophase, but this was not so in the short-winged morph (Zera and Cisper, 2001; Zhao and Zera, 2004). Such diurnal variation was also found in natural populations (Zera *et al.*, 2007), and thus cannot be ascribed to laboratory conditions and selection. Furthermore, it persisted under constant darkness and was temperature compensated, thus qualifying as a genuine circadian rhythm within the endocrine system (Zera and Zhao, 2009). Allatostatin-like material was shown to also exhibit a diurnal modulation in long-winged crickets, but not in short-winged ones (Stay and Zera, 2010), thus qualifying as a potential upstream regulatory factor.

Genetic variation that could explain such threshold mechanisms has been found in laboratory strains and natural populations of *G. firmus,* making this a prime model system to investigate functional causes of adult life history evolution in the context of evolutionary endocrinology (Zera, 2006, 2007).

11.2.2.2. Phase polyphenism in locusts Locust phase polyphenism (a switch between solitary and gregarious forms) has dramatically impacted human history. The gregarization phenomenon can lead to staggering densities of animals, and these migratory swarms of locusts are one of the world's most devastating plagues (for historical and recent data on locust pest status see Pener and Simpson, 2009; Sword *et al.*, 2010). Phase polyphenism is common in several species of locusts, but is most clearly expressed and best studied in the migratory locust (*Locusta migratoria*) and the desert locust (*Schistocerca gregaria*).

Phase polyphenism is a complex phenomenon that involves changes in body coloration, wing morphology, reproductive physiology, energy metabolism, and behavior (Uvarov, 1921). Solitary phase animals (instead of solitary, the term "solitarious" is also frequently used in the scientific literature on locusts to resolve possible ambiguities in the term solitary, but for reasons of simplicity we prefer here to speak of solitary phase locusts, also when referring to laboratory animals reared in isolation) generally avoid conspecifics and have cryptic or green color patterns and reduced wing morphologies and musculature. Gregarious phase animals aggregate, actively seeking conspecifics, and have a dark background pigmentation with a frequently yellow or orange color pattern, as well as more fully developed wings and wing musculature. The complexity of this syndrome sets locust phase polyphenism apart from the apparently simpler wing polyphenisms already discussed for other hemimetabolans (including other orthopterans). Perhaps the most important difference between locust phase polyphenism and the other polyphenisms discussed so far is that the full transition from the solitary to the gregarious form generally requires multiple generations, and thus a transgenerational route of information transfer.

Gradual phase transition from solitary to gregarious locust forms was one of the earliest of the studied types of polyphenisms (Uvarov, 1921) and for decades the endocrine system, and in particular, JH, was advocated as the main control center (Couillaud *et al.*, 1987; Joly, 1954; Staal and de Wilde, 1962). However, results from many of these early hormone studies were equivocal, and the role of JH continues to be a controversial issue (Applebaum *et al.*, 1997; Dorn *et al.*, 2000; Pener, 1991; Pener and Yerushalmi, 1998). Fortunately, a series of hormone titer studies and their interpretation in an organismic context has now resolved several of the discrepancies, not only for JH but also as for other endocrine system functions.

Before going into further detail we call attention to two recent reviews, the one by Pener and Simpson (2009), which addresses the full complexity of locust phase polyphenism in an impressive 286 pages, and a more concise review by Verlinden *et al.* (2009). These reviews were helpful in revising some of the considerations on the hormonal control of locust phase polyphenism expressed

in our previous version of this chapter (Hartfelder and Emlen, 2005).

11.2.2.2.1. Changes in color and morphology The most important cue leading to phase change is the level of crowding experienced by nymphs as they develop. Sensory excitation of hindleg mechanoreceptors has been identified as a powerful stimulus to elicit behavioral and gradually also other phase characteristic changes (Simpson *et al.*, 2001). Models built from particle physics properties showed that tactile stimuli to the hindlegs elicits self-organization of crowded locusts into marching hopper (nymph) bands and gregarious flight movement of adults (Buhl *et al.*, 2006). The driving force behind this self-organization is a simple rule, avoidance of cannibalism from behind (Bazazi *et al.*, 2008). A strong correlation was demonstrated between hindleg bristle mechanostimulation, gregarious behavior, and serotonin levels in thoracic ganglia (Anstey *et al.*, 2009), which is evidence for the transformation of the population density signal into a fast (early) endogenous response, but first leading to a switch in behavioral state.

Color differences between gregarious and solitary locusts are the best known and most visible phase characteristic (**Figure 3**, lower panel). There are separate kinds of color polyphenism in acridids; namely a phase color polyphenism expressed as dark body pigmentation or not, a green-brown background color polyphenism, homochromy (i.e., adaptation of the body color to the respective background), and a yellow/orange background color which, in combination with dark body patterns, is frequently interpreted as having an aposematic function (Sword, 2002). Different locust species may vary in the degree of expression of these color variations. Furthermore, within a species they may vary with respect to environmental conditions, especially humidity. Also

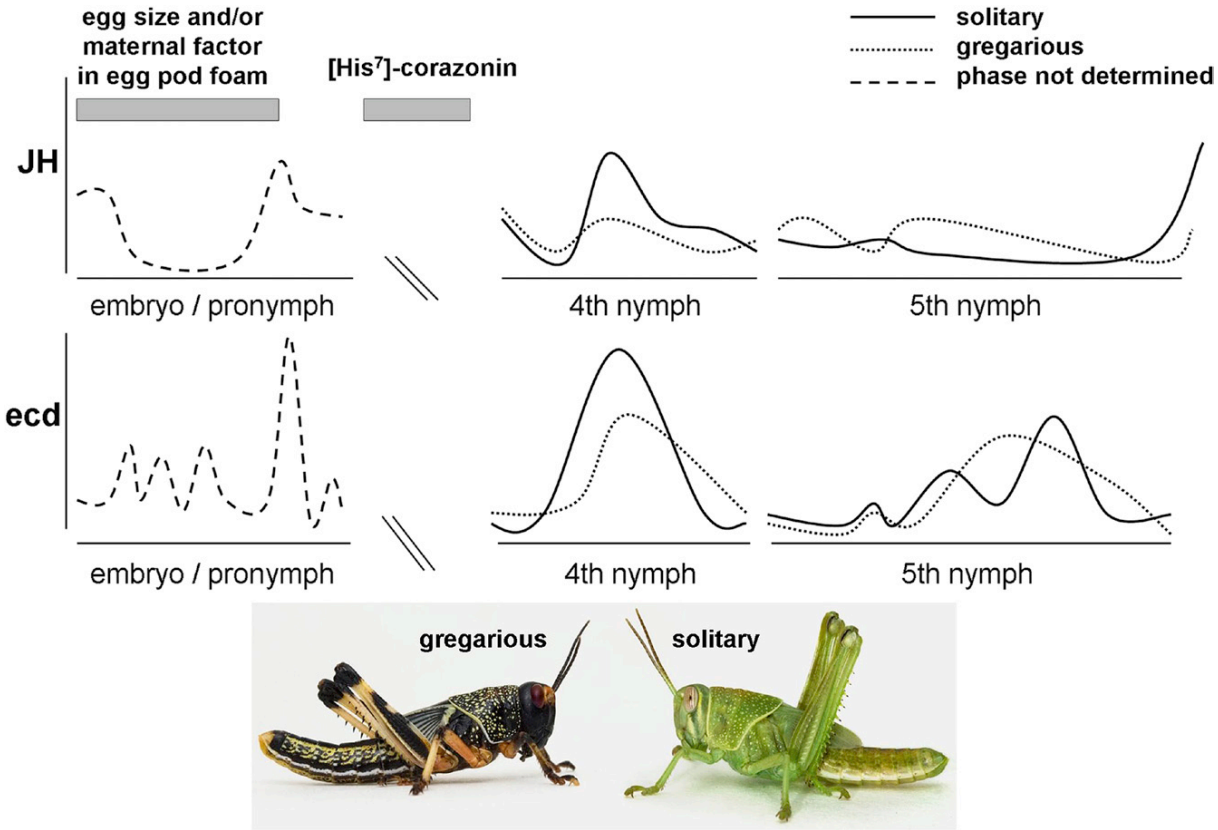

Figure 3 Juvenile hormone and ecdysteroid titers during embryonic and post-embryonic development of solitary and gregarious morphs of migratory locusts (*Locusta migratoria* and *Schistocerca gregaria*). Hormone titers during embryonic development were determined by *Lagueux et al.* (1977) and were not specified with respect to phase. The ecdysteroid titer peaks during embryonic development are associated with embryonic molts and the JH-free period in the middle of embryonic development is required for correct blastokinesis (*Truman and Riddiford*, 1999). Larval hormone titers compiled in this graph were determined by *Botens et al.* (1997), *Tawfik et al.* (1997b), and *Tawfik and Sehnal* (2003). JH application to gregarious/crowded fourth instar larvae was shown to shift several of the solitarious traits (especially green color), but did not shift the entire set that characterizes the gregarious morph (see text). Corazonin application to isolated/solitary second and third instar larvae induced the gregarious-phase dark foreground coloration (Tanaka, 2000a,b,c). Small egg size and/or exposure of eggs of isolated/solitarious females to a maternal factor present in egg pod foam from gregarious/crowded females shifts offspring to the gregarious phase (*Häegele et al.*, 2000; *Tanaka and Maeno*, 2006). Typical color variants for gregarious and solitarious morphs of *S. gregaria* are shown in the lower panel. Photo copyright Tom Fayle.

nymphs and adults may be different in these color combinations, in part due to the transgenerational gradual transition between the phases.

Exposure to crowded conditions induces a shift from the solitary (frequently green) to the gregarious (brown/black with yellow, *S. gregaria*) or orange (*L. migratoria*) form, and much of this shift is mediated by hormones. Functionally, the best characterized hormone is the neurohormone Lom-DCIN, originally identified in studies on an albino mutant of an *L. migratoria* strain (Tanaka, 1993). This neurohormone has since been chemically characterized as [His^7]-corazonin (Tawfik *et al.*, 1999a) and shown to induce the expression of the black pigmentation and foreground color patterns typical of the gregarious form (Pener *et al.*, 1992; Tanaka, 2000a–c, 2001). Schoofs *et al.* (2000) found immunoreactive staining to a DCIN in lateral neurosecretory cells and in corpora cardiaca (CC), as well as in a few other distinct neurons. Subsequently, the detection of DCIN in both gregarious and solitary phase nymphs (Baggerman *et al.*, 2001) indicated that its release may be blocked in solitary ones.

Whereas [His7]-corazonin is established as the prime factor involved in the expression of the dark pigmentation of the gregarious form, JH is a major effector leading to the green (or better to say, not brown) background coloration. As stated by Pener and Simpson (2009):

In locust species that exhibit green-brown colour polyphenism, the green colour inducing effect of implantation of extra CA, or administration of JH or JH analogues (JHAs), has been repeatedly confirmed without any exception. These treatments induce green colour even in crowded locust hoppers that show a reduction or disappearance of the gregarious colouration with the increasing green colour.

It is now recognized that the solitary–gregarious phase transition is also associated with changes in a suite of morphological traits, including aspects of wing morphology and relative wing size (Dorn *et al.*, 2000), differences in morphometric ratios related to the hindleg femur (for review see Pener and Simpson, 2009), and especially the number of ovarioles. The number of ovarioles, which is already determined in the embryonic stage, is higher in solitary than in gregarious females (Pener, 1991) causing fertility differences between the morphs, contributing to a larger number of eggs per egg pod in the solitary morph. As this difference in ovariole number is also associated with the size of the eggs, which are smaller in solitary locusts, these traits are indicative of major phase differences related to female reproductive strategies.

Whereas JH and [His^7]-corazonin certainly play major roles in pigmentation changes, their role in shaping the entire suite of phase characters is far from clear, despite a plethora of experimental studies (for a comprehensive review of discrepancies in results and interpretations see Pener and Simpson, 2009). It is noteworthy at this point that the solitary phase characteristics cannot be interpreted as an effect of juvenilization (or neotinization) simply due to higher JH titers. Nevertheless, measurements of endogenous hormone titers are crucial to any interpretation of results obtained by experimental manipulation of hormone levels, either by gland extirpations or transplantations, or topical application of hormones or pharmacological analogs (Zera, 2007).

11.2.2.2.2. Juvenile hormone and ecdysteroid titers of solitary and gregarious nymphs In a pioneering study, Injeyan and Tobe (1981) showed that during the fourth nymphal instar, the CA were more active in animals reared under isolated conditions than they were in animals reared under crowded conditions. Subsequently, Botens *et al.* (1997) compared JH III titers measured for laboratory animals reared under isolated and crowded conditions. These authors noted that the JH hemolymph titer was higher in fourth (penultimate) instar nymphs reared in isolation (solitary) when compared to crowded ones, but such differences were no longer encountered in last instar nymphs. Furthermore, Botens *et al.* (1997) also measured JH III titers for wild-caught solitary and gregarious animals and found a similar result; JH titers during the middle of the fourth nymphal instar were higher in solitary animals than in gregarious ones. These findings are consistent with a critical window for JH action during nymphal development (**Figure 3**). In addition to JH III, which is the principal form of JH in *L. migratoria* (Bergot *et al.*, 1981), Darrouzet *et al.* (1997) found that the CA of *L. migratoria* also synthesize two hydroxyjuvenile hormones (12′-OH JH III and 8′-OH JH III). These products have not been detected by the standard radiochemical assays (Tobe and Pratt, 1974), and whether these are secreted into the hemolymph or whether they affect phase polyphenism remains to be explored.

Solitary and gregarious locusts also differ in their 20E levels (**Figure 3**). Animals reared under crowded conditions have a slightly lower but more prolonged premolting ecdysteroid peak than animals reared under solitary conditions in the penultimate and last nymphal instars (Tawfik *et al.*, 1996; Tawfik and Sehnal, 2003). As these differences coincide with the phase specifically modulated JH titer in the fourth instar, a synergistic interaction of these major morphogenetic hormones is feasible, even though a clear morphogenetic role for ecdysteroids in locust phase polyphenism has not yet been shown.

11.2.2.2.3. Changes in adult behavior and physiology Adult solitary and gregarious locusts differ in behavior and physiology, as well as in morphology. For example, gregarious animals show an affinity for other locusts (aggregative behavior) and a strong propensity for long-duration migratory flights, in contrast to solitary animals. These behavioral differences are accompanied by corresponding differences in adult physiology related to the two different

life-history strategies. Phase differences are noticeable in the strength of the adipokinetic reaction that mobilizes fat body lipids for long-distance flight (see Pener and Simpson, 2009 for a comprehensive discussion on the role of adipokinetic hormone in locusts) and at the onset of egg production, with crowded females generally beginning egg production slightly earlier than isolated ones (Tawfik *et al.*, 2000).

As with the morphological differences previously discussed, behavioral differences between solitary and gregarious forms also appear to be driven, at least in part, by hormones. In adult females, both solitary and gregarious animals show a major increase in JH levels within the first two weeks after eclosion (Dale and Tobe, 1986; Tawfik *et al.*, 2000). However, this rise in JH occurs earlier in solitary females than it does in gregarious ones (Dale and Tobe, 1986; Tawfik *et al.*, 2000). Similar differences in the timing of hormone secretion were observed for ecdysteroids, with ecdysone levels rising earlier in solitary females than in gregarious ones (Tawfik and Sehnal, 2003; Tawfik *et al.*, 1997b).

Even though the role of JH in the induction of vitellogenin synthesis in locusts is a well-established model in insect physiology (Nijhout, 1994), these phase polyphenism-related differences in the timing of adult female JH and ecdysteroid synthesis and titers are not easily reconciled with phase-specific differences in the timing of the first oogenic cycle (**Figure 4**), as females raised under crowded conditions begin producing eggs slightly sooner than females reared under isolated conditions (Tawfik *et al.*, 1997a, 2000). The hormone titer differences, however, may be related to migratory flight fuel metabolism (Wiesel *et al.*, 1996). Low levels of JH (as in gregarious females) stimulate utilization of stored lipids for flight, whereas high levels of JH (as in solitary females) reduce their use, and instead, result in vitellogenin synthesis and lipid allocation to oocytes (Wiesel *et al.*, 1996).

In adult males, the principal behavioral difference between solitary and gregarious phase individuals involves production and secretion of an aggregation pheromone that has phenylacetonitrile (PAN) as its main component (Mahamat *et al.*, 1993). Tawfik *et al.* (2000) showed that pheromone emission by crowded adult males starts between days 10 and 15 after fledging, coinciding with a conspicuous increase in the amount of JH III in the hemolymph. As pointed out by Pener and Simpson (2009), the term aggregation pheromone may not entirely be correct, and due to its repellent effects on conspecific males they

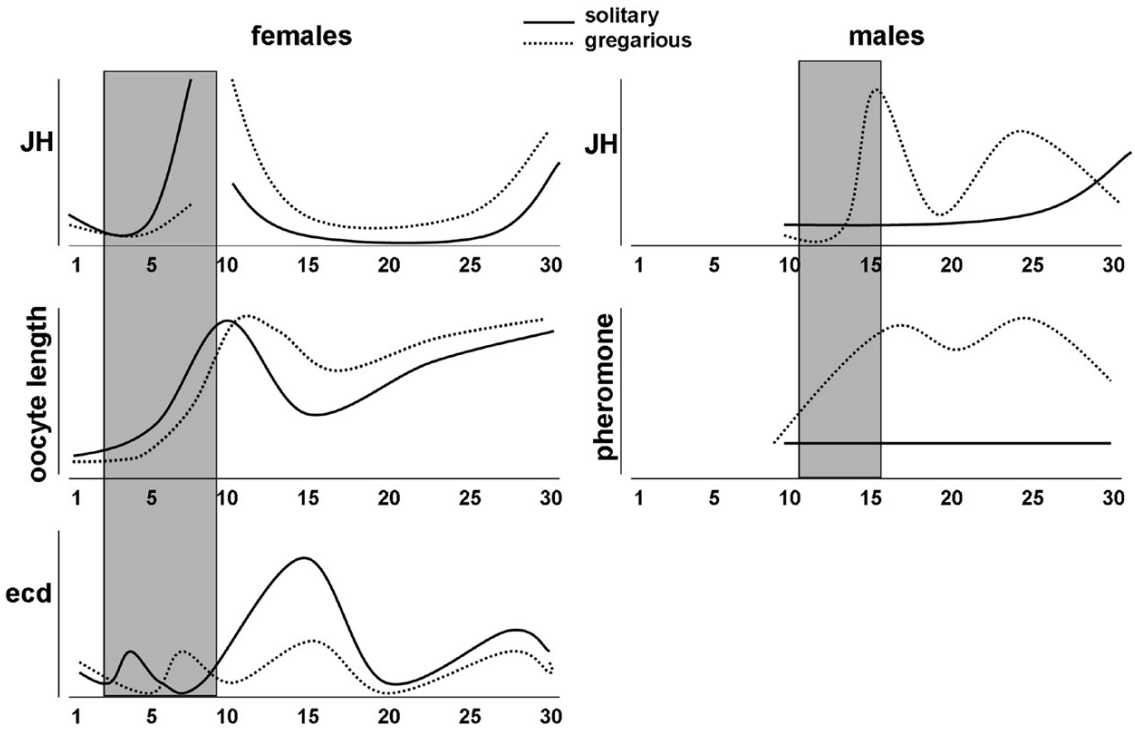

Figure 4 Hormone titers in relation to terminal oocyte length in solitary and gregarious-phase locust females (*Locusta migratoria* and *Schistocerca gregaria*), and the phase relationship between JH titer and pheromone production (phenylacetonitrile) in adult males. The phase differences in the JH and ecdysteroid titer of females are reflected in an earlier onset of growth of the terminal oocytes in solitarious females, nevertheless, laying of the first batch of eggs occurs somewhat earlier in gregarious females, possibly due to smaller egg size. In gregarious-phase males, the sharp increase in the JH titer seen between days 10 and 15 coincides but may not necessarily be causally related to the strongly enhanced production of a gregarization pheromone. Graph compiled from data by *Tawfik et al.* (1997a,b, 1999a, 2000), *Tawfik and Sehnal* (2003), and *Dorn et al.* (2000).

propose the term "rival male repelling pheromone." Since a chemically not yet defined maturation-accelerating effect of mature *S. gregaria* males on immature ones has long been noted and appears to be mediated via the CA (Loher, 1961), the transient steep rise in the hemolymph JH titer seen in young males (**Figure 4**) may be more related to the coordination of maturation among gregarious males, and less so with PAN production, especially since the latter has been shown to quickly respond to changes in population density (Deng *et al.*, 1996).

11.2.2.2.4. Maternal effects on offspring development *Schistocerca gregaria* females reared in isolation do not only lay more eggs than those reared under crowded conditions, due to the larger number of ovarioles, but these also lay smaller ones. This was also observed for the first egg pod laid by a female reared under crowded conditions (Maeno and Tanaka, 2008). Egg size is therefore considered to be an important transgenerational cue (Tanaka and Maeno, 2010).

Egg development is influenced greatly by ovarian ecdysteroids, and by measuring these Tawfik and Sehnal (2003) and Tawfik *et al.* (1999b) found that ecdysteroid contents of ovaries of females (*S. gregaria*) reared under crowded conditions were up to four times higher than those in the ovaries of females reared in isolation (8.9 ng/mg vs. 2.3 ng/mg tissue before egg laying). These phase-specific differences in egg ecdysteroid levels persisted after the eggs were laid (89 ng vs. 14 ng per egg), and even were reflected in newly hatched larvae (Tawfik *et al.*, 1999b). High levels of ecdysteroids have long been detected in vitellogenic ovaries (Lagueux *et al.*, 1977), where they are synthesized by the follicle epithelial cells. These ecdysteroids are transferred to the developing eggs and, together with JH, they affect molting events during embryonic development (Lagueux *et al.*, 1979; Truman and Riddiford, 1999). These studies, thus, raise the possibility that endocrine differences in the mothers, resulting from their exposure to crowding as they developed, are carried over to their offspring. This may explain some aspects of the transgenerational nature of this phase transition.

Pheromones and semiochemicals transferred to eggs may also direct embryonic development. These compounds, when deposited on the eggs or egg pod material, not only attract other gravid females to the area resulting in clustered oviposition (Saini *et al.*, 1995), but they may also lead to an increase in the propensity of the hatchlings to express gregarious characteristics. Washing freshly laid eggs from gregarious *S. gregaria* females shifted phase characteristics from gregarious toward solitary, and application of female accessory gland products to these washed eggs restored expression of the gregarious characters (Hägele *et al.*, 2000; McCaffery *et al.*, 1998). These findings have been challenged by Tanaka and Maeno (2006), but a recent study on *L. migratoria* (Ben Hamouda *et al.*, 2009) observed an effect similar to that seen by Hägele *et al.* (2000). A possible reason given by Pener and Simpson (2009) for the discrepancy in the findings on *S. gregaria* egg foam activity could be a genetic difference in the strains used. Nevertheless, when testing egg foam effects in different strains, this hypothesis was not confirmed (Maeno and Tanaka, 2009), and instead, these authors proposed other factors for predetermination of the hatchling phase fate, such as the egg-size dependent amount of egg yolk. While the chemical nature of the egg-foam maternal agent has been tentatively identified as an alkylated L-DOPA analog (Miller *et al.*, 2008), its role and mode of action continues to be a matter of debate (Tanaka and Maeno, 2010). Nonetheless, the existence of a transgenerational transmission of phase characteristics is unquestionable.

A phase-related 6 kDa molecule has been identified from a proteomic screen on hemolymph of *S. gregaria* reared under crowded conditions (Rahman *et al.*, 2003). Interestingly, the strongest immunoreactivity for this peptide was found in follicle cells of the ovary and in seminal vesicles of the male accessory gland complex (Rahman *et al.*, 2008), thus opening the possibility that it may be transmitted to females during copulation and be part of the transgenerational phase determination process.

11.2.2.2.5. Genomic resources Studies comparing patterns of protein and gene expression patterns between solitary and gregarious animals were initiated at the turn of the century (Clynen *et al.*, 2002; Rahman *et al.*, 2003; Wedekind-Hirschberger *et al.*, 1999), but only with the generation of a cDNA library and the sequencing of 76,012 ESTs clustered into 12,161 unique sequences (Kang *et al.*, 2004) did high throughput, large-scale analysis become feasible, The transcriptome comparisons of solitary and crowd-reared *L. migratoria* nymphs revealed over 500 differentially expressed genes, 70% of these represented novel transcripts without similarity in non-redundant databases. Two subsequent studies by this group (Guo *et al.*, 2010; Wei *et al.*, 2009) characterized differentially expressed small RNAs and transcripts of transposable elements, respectively. The most abundant one of the transposable element transcripts was cloned and turned out to be differentially expressed in the nervous system, making it a possible mediator in phase-specific neural responses.

An alternative to non-hypothesis-driven, high throughput studies are candidate gene approaches. Special attention has been given to neuroparsins, initially isolated from CC of *L. migratoria* (Girardie *et al.*, 1987) and genes encoding components of the insulin signaling pathway. Monitoring transcript levels of two neuroparsins, Scg-NPP3 and Scg-NPP4, in brain and abdominal tissue revealed phase-dependent modulation in adult locusts (Claeys *et al.*, 2006). Badisco *et al.* (2008) quantified

mRNA levels of an insulin-related peptide (Scg-IRP) in adult *S. gregaria* and detected phase-dependent differences in the fat body. Furthermore, they showed that a recombinant Scg-NPP4 peptide was capable of binding Scg-IRP, inferring a cross-talk between these signaling pathways during sexual maturation.

11.2.3. "Isoptera" (Termites) — Caste Polyphenism in the Hemimetabola

The characterization of termites as "social cockroaches" (Korb, 2008) already hints at the very special position of termites within insects expressing caste polyphenism, even more so as the order Isoptera has recently literally been relinquished to the status of a clade nested within the cockroaches (Blattodea; Inward *et al.*, 2007). Caste polyphenism in termites differs from that of the holometabolous Hymenoptera in three very important ways. First, all isopteran species are social, whereas sociality has evolved in only a few branches of the Hymenoptera. Second, caste polyphenism in termites is a larval polyphenism, that is, it primarily affects the morphologies and behavior of larvae, rather than adults. Finally, in termite societies both males and females form castes, and thus equally contribute to the social organization. In contrast, hymenopteran societies are all female based.

Hemimetabolous development permits post-embryonic stages to actively participate in termite colony life, and these post-embryonic larval and nymphal stages constitute the major work force of termite colonies (in termites, the early post-embryonic instars that are frequently dependent on being cared for are called larvae, whereas later instars are called nymphs).The only true imagoes encountered in a termite colony are the sexuals, — primary reproductives (king and queen), and, at certain times of the year, the pre-dispersal sexual alates. In the adults, there is little visible dimorphism between the sexes, and the differences that exist primarily result from the high degree of ovarian activity leading to physogastry in the egg-laying queen (Bordereau, 1971). In contrast, many termites exhibit marked sexual dimorphism in the larval stages, especially in taxa that show a sex bias with respect to caste phenotypes. For termite sociality, the spotlight is thus on the polyphenism exhibited by the larval/nymphal stages.

Larval/nymphal polyphenism means that in subsequent developmental stages an individual can progressively specialize for different colony tasks ("temporal polyphenism" according to Noirot and Bordereau, 1988). As such, caste polyphenism is tightly linked to the molting process and to the endocrine factors regulating molting. Interestingly, molting in termites is no longer necessarily connected with growth (Noirot, 1989), and has instead been co-opted as a mechanism for polyphenic changes in animal shape.

Reviews on termite societies and trajectories of caste development within these (Korb and Hartfelder, 2008; Nalepa, 2009; Noirot, 1985a,b, 1989, 1990; Noirot and Pasteels, 1987; Roisin, 2000) emphasize the distinction between caste systems (and polyphenic mechanisms) in "lower" (Mastotermitidae, Kalotermitidae, Hodotermitidae, and Rhinotermitidae) and "higher" termites (Termitidae) and their relationship to nesting modality and nesting substrate types. Lower and higher termites differ primarily in the development of the worker caste. In the higher termites, workers represent a clear developmental trajectory, culminating in a terminal molt. In contrast, in the lower termites, a true worker caste is rare. Instead, most of the nymphal stages perform worker functions, such as colony maintenance. Workers in the lower termites do not undergo a terminal molt, and these individuals retain the capacity to subsequently develop into either soldiers or reproductives, or simply remain workers. In the lower termites, these late instar nymphs that comprise the major work force of a colony are generally described as false workers or pseudergates (for explanations on termite-specific terminology for developmental stages and their roles in termite societies see Korb and Hartfelder, 2008).

Caste polyphenism in termites involves several possible developmental switches, such as larva to pre-reproductive (with wingpads) to reproductive, larva to presoldier to soldier, and, in the higher termites, larva to worker. These developmental transformations may require several subsequent molts and several different critical periods (**Figure 5**). Several of these developmental transformations can be reversed midway through the process, permitting extraordinarily flexible adjustment of the production of castes in the regulation of colony structure. This is particularly common in the lower termites, which are famous for undergoing stationary, and even regressive molts.

11.2.3.1. The pathways leading to reproductive development Termite nymphs occasionally molt into reproductives. The most commonly encountered reproductives are the alates — the winged males and females that disperse from termite colonies to breed and found new colonies. After mating, each new royal pair sheds its wings and founds a colony. A second type of reproductive consists of a replacement king or queen within an existing colony. When colonies bud, or when one of the original members of the royal pair dies, replacement reproductives can be produced. In either case (dispersing alates or replacement reproductives), commitment toward a sexual fate is first evidenced by the appearance of rudimentary wingpads, which may appear several molts before the terminal, adult molt. However, in the developmental trajectory of "replacement" reproductives, these wingpads fail to fully develop, so that replacement kings or queens do not have functional wings. Termites

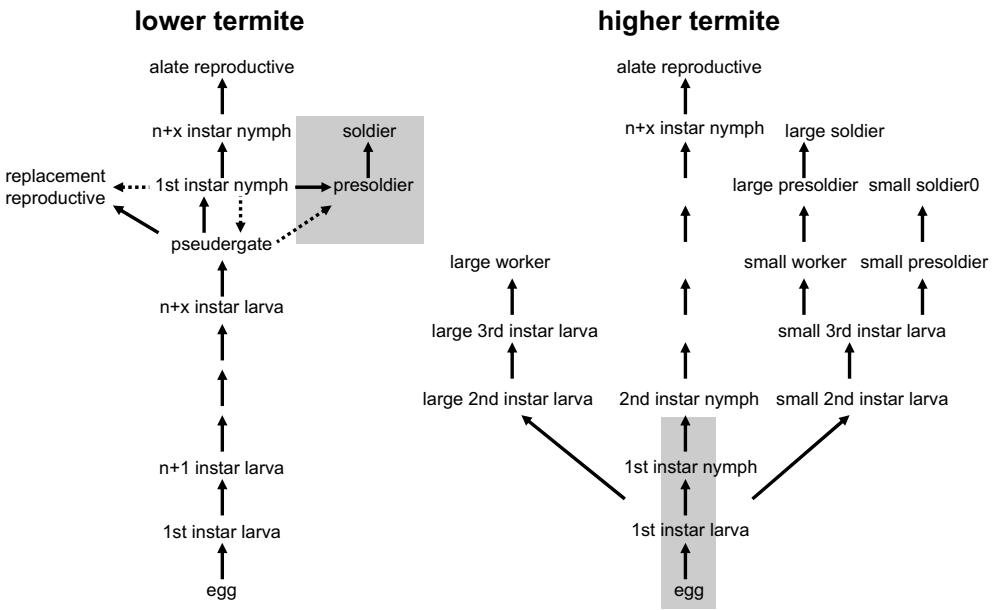

Figure 5 Generalized developmental pathways in lower and higher termites. In termite studies it is customary to denote all immatures stages without wingpads as larvae. Nymphs are stages exhibiting wingpads and thus potentially develop into sexual alates. Lower termites pass through a variable number of larval stages before the last nymphal stage (frequently referred to as the pseudergate stage), which is the main branching point for nymphal to alate sexual (imaginal) development, to neotenic replacement reproductives, or to soldiers. In most primitive termites there is no true worker caste, since tasks of colony maintenance are performed by immatures that still retain several developmental options. In higher termites, the branchpoint for developmental pathways is set early in larval development, leading alternatively to the nymphal/adult line, the soldier line, and to a true, definitive worker caste. Shading emphasizes nodes where hormone titers (JH and/or ecdysteroids) differ between castes, or where JH applications bias development, principally into presoldier/soldier differentiation. Modified from *Noirot* (1990), *Miura* (2004), *and Korb and Hartfelder* (2008).

that do not develop wingpads during any of these decisive molts are committed to remain in the work force or to become soldiers.

Interestingly, the number of larval/nymphal instars that an individual completes prior to becoming a reproductive or a neuter (worker or soldier) differs considerably among species. In the higher termites, this decision usually occurs during the first or second instar, while lower termites may pass through six or more pre-commitment molts (**Figure 5**).

Environmental triggers for termite reproductive caste development primarily involve demographic aspects of the colony, as communicated through chemical signals transmitted from workers to larvae (Noirot, 1990). Most early work on this polyphenism focused on the differentiation of *Kalotermes flavicollis* nymphs after removal of the primary reproductive pair (Lüscher, 1964; Wilson, 1971). Once the king or queen had been removed, nymphs began developing into replacement reproductives, suggesting that levels of chemical signals from the primary pair triggered the switch between non-reproductive and reproductive development. These studies led to the now classical model of negative feedback, where the king and queen each secrete compounds that repress the development of replacement reproductives of their respective sex (Lüscher, 1964). These signals are thought to act via the neuroendocrine system (Lüscher, 1976).

While this classical model had its ups and downs during the last decades due to a lack of empirical evidence on the chemical nature of such pheromones, a major breakthrough has come from the termite *Reticulitermes speratus* for which a volatile inhibitory pheromone produced by female neotenics has been identified (Matsuura *et al.*, 2010). Interestingly, its active compounds are also released from eggs, inferring that reproductive status and inhibitory power are tightly linked.

Unfortunately, the hormonal control of termite reproductive development is not well understood. This is due in part to the complexity of the polyphenism (many different "switches" are involved, and several of these have multiple critical periods spread over several subsequent molts), and in part due to the lack of sensitive bioassays for the activity of termite endocrine glands. When monitoring *in vitro* CA activity for *Zootermopsis angusticollis,* the pheromonal secretions of the royal pair were denoted as inhibiting JH biosynthesis rates, inferring a link between pheromonal cues and polyphenic regulation of larval/nymphal development (Greenberg and Tobe, 1985). Similar findings come from the damp-wood termite *Hodotermes sjostedti*, where a JH peak normally observed during molting

events was absent in the one leading up to imaginal differentiation (Cornette *et al.*, 2008). Subsequently, as nonphysogastric nymphoids developed into queens, JH titers were observed to increase, preceding the progression of vitellogenesis in another *Reticulitermes* species, thus building evidence for a major role of JH in female reproductives (Maekawa *et al.*, 2010).

The physiological basis underlying seasonal alate production is more difficult to ascertain because production of alates depends on seasonal factors and not just the presence or absence of the social pair. However, studies of Lanzrein *et al.* (1985) suggested that termite queens may transfer hormones to their eggs, and in this fashion affect the production of alate reproductives. Specifically, they found that in two *Macrotermes* species, queens transferred both JH and ecdysteroids to their eggs, and elevated levels of these hormones were found during embryonic development in larvae biased toward becoming alate reproductives.

11.2.3.2. Mechanisms underlying soldier development Much more effort has been put into studies of the endocrine regulation of soldier development. Soldier castes are the hallmark of termite societies and may be considered an evolutionary novelty characterizing the clade of "social cockroaches." Soldiers have heavily sclerotized exoskeletons and specialized structures for colony defense, including enlarged mandibles or enlarged heads that squirt sticky glandular secretions capable of entangling their main predators, ants.

Whereas most of the earlier studies investigated the role of exogenously applied JH as a means to pest control (promoted by offsetting the worker/soldier ratio in colonies), more recent efforts were directed to understanding the endocrinology of the presoldier and soldier molts. Most of these studies were done on diverse species of lower termites, and many of these studies connected field data to controlled laboratory experiment designs. JH III titers in presoldier stages of the Formosan subterranean termite *Coptotermes formosanus*, a major pest species, were significantly higher than those of workers or soldiers, denoting the important role of JH in this transition stage of soldier development both in field samples (Liu *et al.*, 2005a; Park and Raina, 2004) and under laboratory conditions varying temperature and nutrition (Liu *et al.*, 2005b). A subsequent study comparing CA activity in *R. flavicollis* showed that workers developing into presoldiers had 2.5-fold higher JH synthesis rates than those developing into neotenic reproductives (Elliott and Stay, 2008).

Most important, colony size and its worker/soldier ratio turned out to be crucial in stimulating or inhibiting competent larval stages to enter the presoldier–soldier route through modulating JH titers (Mao and Henderson, 2010). Furthermore, live soldiers or soldier head extracts were shown to inhibit soldier development and the transcriptional profile associated with this pathway in *R. flavipes* (Tarver *et al.*, 2010).

The most thought-provoking results on a key mechanism underlying termite caste development, however, came from studies on the modulation of two major larval storage proteins hexamerin 1 (Hex1) and hexamerin 2 (Hex2) in *R. flavipes* (Scharf *et al.*, 2005a). Sequence analysis and functional assays indicated that (1) Hex1 is capable of covalently binding circulating JH, thus sequestering it from the biologically active hormone pool, and (2) Hex2 expression is contingent on JH levels (Zhou *et al.*, 2006a,b). This hexamerin-intrinsic circuitry favors soldier development when the Hex1/Hex2 ratio is low. In contrast, Hex1 accumulation in well-fed colonies and an appropriate worker/soldier ratio inhibited the development of new soldiers (Scharf *et al.*, 2007). This not only establishes a unique link between extrinsic conditions (nutrition, colony composition) and the intrinsic JH titer through hexamerins, it also directly affects downstream JH-responsive genomic networks, as shown by hexamerin RNAi (Zhou *et al.*, 2007).

11.2.3.3. Termite genomics and new frontiers Taken together, these recent insights on endocrine system-mediated regulatory mechanisms underlying sexual (alates and secondary reproductives), worker (false/pseudergate or true workers), and soldier development now have little in common with hormone-application-derived models on caste development, which postulated distinct critical periods for polyphenic switching between termite castes, an early one for the nymphoid/alate pathway, and a later one for the worker/soldier decision.

Apart from a recent revival in endocrine studies, the quest for understanding caste differentiation in termites has gained new impetus from studies on differential gene expression. The first studies were directed at understanding the soldier developmental pathway (Miura, 2001; Miura *et al.*, 1999), leading to the identification of a gene with soldier-specific expression in the mandibular gland of the damp-wood termite *H. japonica*. This gene encodes a novel protein (SOL1) with a putative signal peptide, indicating that it may be a soldier-specific secretory product of this gland. The gland develops from a disc-like structure once a presoldier-differentiating molt has been induced by a high JH titer (Miura and Matsumoto, 2000; Ogino *et al.*, 1993). Subsequent DNA macroarray studies on *R. flavipes* set up to reveal the molecular underpinnings of reproductive caste development denoted 34 nymph-biased genes (Scharf *et al.*, 2005b), including those functionally related to vitellogenesis and JH sequestration. More recent differential gene expression screens identified gene sets related to the development of neotenics in the dry-wood termite *Cryptotermes secundus* (Weil *et al.*, 2007). With sequencing efforts on termite genomes underway, the results of these pioneering studies should address gene network

questions built around environmental and endocrine factors in termite caste development.

Yet these certainly important genomic insights probably will do little to resolve a major enigma in termite development, namely the switches in molting types in lower termites, from progressive to stationary and even regressive molts. The latter are a major puzzle to insect physiologists. Endocrine signatures underlying these molting types are now emerging from mass hormone titer assaying in two species, *H. sjostedti* (Cornette *et al.*, 2008) and *C. secundus* (Korb *et al.*, 2009) indicating that relative JH and ecdysteroid titer dynamics during the late nymphal stages are crucial to predicting the outcome of the subsequent molt.

11.3. Polyphenism in the Holometabola

In contrast with the Hemimetabola, wing length polyphenism does not play a prominent role in the Holometabola. In this group, the prevalent forms of polyphenism are related either to camouflage, such as in color and wing pattern polyphenism in butterflies, to reproductive strategies, such as the development of weaponry in male stag and rhinoceros beetles, or to reproductive division of labor as seen in the female castes of many Hymenoptera.

11.3.1. Lepidoptera

11.3.1.1. Pupal color polyphenism Lepidopteran pupae display a remarkable crypsis; this immobile life stage is especially vulnerable to predators, and pupae rely on hiding for survival (Baker, 1970; Hazel, 1977; Hazel *et al.*, 1998; Sims, 1983; West and Hazel, 1982, 1985; Wicklund, 1975). Most butterflies pupate in the soil or leaf litter, and pupae are generally constitutively brown (West and Hazel, 1979, 1996). However, a number of species crawl out of the leaf litter to pupate on the undersides of leaves or branches. Wandering larvae in these species encounter a variety of substrate "backgrounds," and effective pupal crypsis requires a facultative mechanism of pigment production (Hazel *et al.*, 1998; Hazel and West, 1996; Jones *et al.*, 2007; West and Hazel, 1979, 1982, 1996).

Over a century ago it was observed that some butterfly species switch between light and dark pupal forms (e.g., green vs. brown), depending on the background substrate (Merrifield and Poulton, 1899; Poulton, 1887; Wood, 1867). Today, numerous representatives of four lepidopteran families (Danaidae, Nymphalidae, Papilionidae, and Pieridae) are known to exhibit facultative pupal-color polyphenism. Depending on the specific habitats and pupation-substrates of each species, larvae couple pupal color production with exposure to a variety of external stimuli, such as photoperiod (Ishizaki and Kato, 1956; Sheppard, 1958; West *et al.*, 1972; Yamanaka *et al.*, 2004, 2007), relative humidity (Ishizaki and Kato, 1956; Smith, 1978), background color (Gardiner, 1974; Smith, 1978; Wicklund, 1972), temperature (Yamanaka *et al.*, 2009), and background texture and substrate shape/size/geometry (Hazel, 1977; Hazel and West, 1979; Sevastopulo, 1975).

Pupal color polyphenism appears to be regulated by a threshold (Hazel, 1977; Sims, 1983). Although a continuous range of pupal phenotypes are possible (West *et al.*, 1972), natural populations tend to be dimorphic for pupal color, and genetic studies indicate heritable differences among populations for the sensitivity of animals to background substrate characteristics (Hazel, 1977; Hazel and West, 1979; Sims, 1983).

Pre-pupal larvae pass through a "sensitive period" (West and Hazel, 1985) when environmental cues associated with pupation substrate influence the release of a neuroendocrine factor (Awiti and Hidaka, 1982; Bückmann and Maisch, 1987; Hidaka, 1961a,b; Smith, 1978, 1980; Starnecker and Bückmann, 1997). Interestingly, the effect of this factor differs among the lepidopteran families studied.

In Papilionidae, release of this factor (called "browning hormone") results in the production of a dark (brown) pupa; inhibition of release of this factor results in a light (green) pupa, as seen in *Papilio xuthus*, *P. polytes*, *P. demoleus, P. polyxenes, P. glaucus*, *P. troilus*, *Eurytides marcellus,* and *Battus philenor* (Awiti and Hidaka, 1982; Hidaka, 1961a, 1961b; Smith, 1978; Starnecker and Hazel, 1999). In contrast, in Nymphalidae (*Inachis io*), Pieridae (*Pieris brassicae*), and Danaidae (*Danaus chrysippus*), the default pupal color appears to be relatively dark (green), and release of the neuroendocrine factor (pupal melanization-reducing factor; PMRF) stimulates production of a light (yellow) pupa (Maisch and Bückmann, 1987; Ohtaki, 1960, 1963; Smith *et al.*, 1988; Starnecker, 1997). Thus secretion of the neuroendocrine factor has opposite effects on the relative darkness of pupae.

Starnecker and Hazel (1999) extracted the respective neuroendocrine factor from ventral nerve cords of both a nymphalid (*I. io*) and a papilionid (*P. polyxenes*), and injected these neurohormones into sensitive-stage larvae of both the appropriate and the opposite species. In all cases, animals responded to neuroendocrine injections in the species-appropriate way. For example, nymphalid larvae responded to injection of either PMRF (appropriate) or browning factor (inappropriate) by production of light colored pupae, whereas papilionid larvae responded to injection of these same substances by production of dark pupae. Cross-reactivity of these neuroendocrine factors suggests that they are the same factor, and indicates that nymphalid and papilionid butterflies may have independently evolved the capacity to facultatively regulate pupal color (Starnecker and Hazel, 1999). Interestingly,

although these butterfly lineages appear to have co-opted the same neuroendocrine factor, they coupled it with downstream processes of pigment synthesis that are very different (Jones *et al.*, 2007; Starnecker and Hazel, 1999; **Figure 6**).

11.3.1.2. Seasonal wing-pattern polyphenism in butterflies A multitude of butterfly species display seasonal polyphenisms for wing pattern and color. Here we review two of the more common and better characterized of these forms of wing pattern polyphenism: light versus dark hindwings, and presence or absence of ventral forewing eyespots.

11.3.1.2.1. Light versus dark hindwings The most common form of butterfly wing polyphenism involves the overall lightness or darkness of wings. Numerous species in at least four Lepidopteran families are characterized by distinct seasonal light and dark forms, and where it has been studied, this seasonal variation in wing pigmentation results from larval sensitivity to both photoperiod and temperature (Aé, 1957; Endo, 1984; Endo and Funatsu, 1985; Endo and Kamata, 1985; Endo *et al.*, 1988; Hoffman, 1973, 1978; Jacobs and Watt, 1994; Kingsolver, 1987; Kingsolver and Wiesnarz, 1991; Koch and Bückmann, 1987; Müller, 1955, 1956; Nylin, 1992; Reinhardt, 1969; Shapiro, 1976; Smith,

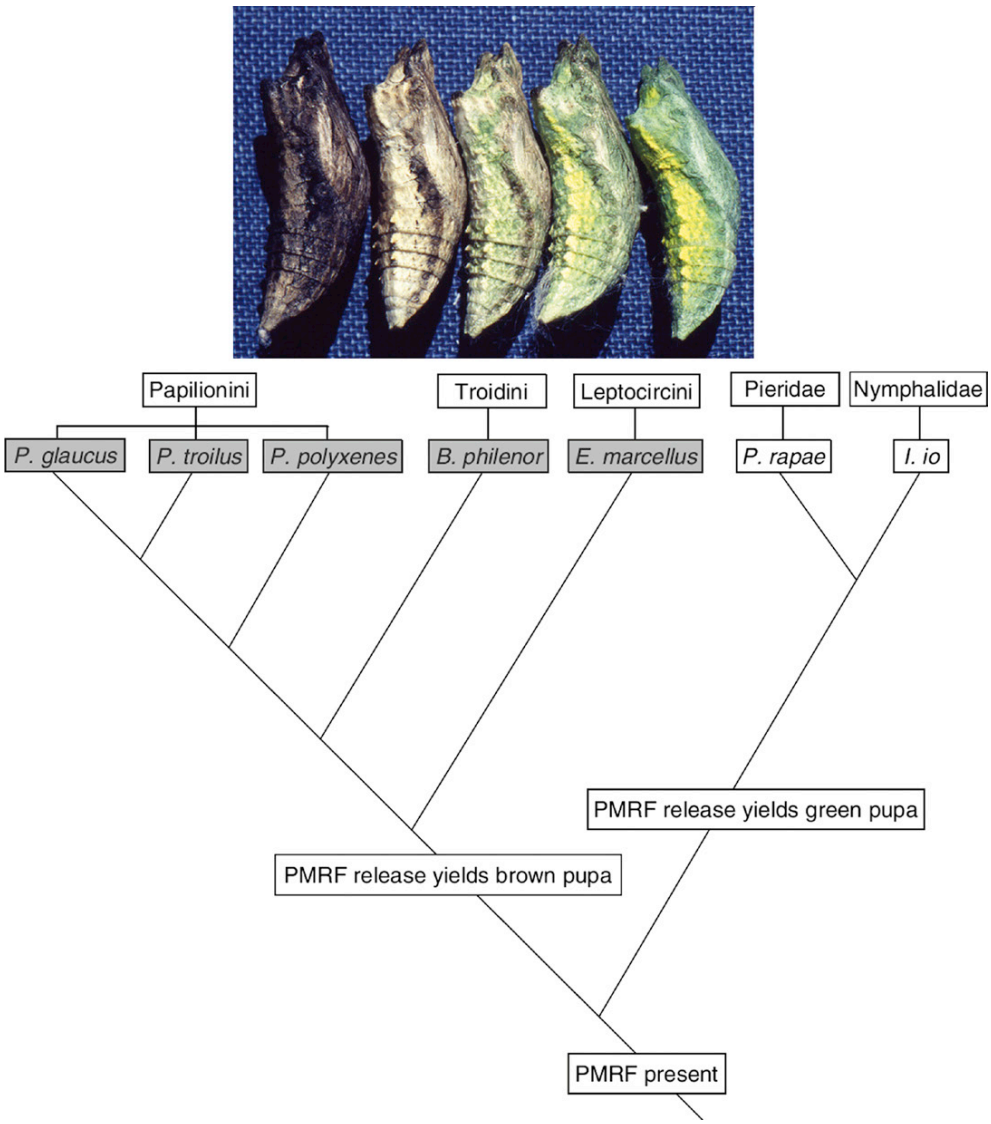

Figure 6 Color polyphenism in lepidopteran pupae. Pupal color polyphenism has arisen multiple times within the butterflies, such as green, orange, and orange-brown pupae of the swallowtail *Papilio xuthus*. (Reproduced with permission from *Yamanaka et al.*, 2004). Where studied, these mechanisms involve an endocrine signal (PMRF) that either darkens or lightens pupal color. A recent comparative study by *Jones et al.* (2007) showed that the Pieridae-Nymphalidae and the Papilionini-Troidini-Leptocircini lineage each evolved pupal color polyphenism independently through co-option of the same endocrine signal. Reproduced with permission from *Jones et al.* (2007). Photos courtesy of Wade Hazel.

1991; Süffert, 1924; Watt, 1968, 1969; Weismann, 1875).

Wing darkness is known to affect the solar absorption and thermal characteristics of butterflies (Jacobs and Watt, 1994; Kingsolver and Watt, 1983; Kingsolver and Wiesnarz, 1991; Watt, 1968, 1969), and natural selection for physiological performance under seasonally variable temperature regimes likely underlies many of these wing-darkness polyphenisms (Karl *et al.*, 2009; Kingsolver, 1987, 1995a,b; Kingsolver and Wiesnarz, 1991). Animals with dark wings are effective at absorbing solar radiation (Kingsolver, 1987; Kingsolver and Watt, 1983; Watt, 1968, 1969). These animals warm quickly, and perform well under cool conditions (e.g., spring), but these same animals are prone to lethal overheating under warmer (e.g., summer) conditions. Most butterfly species with light-dark polyphenisms produce dark forms when animals are reared under short days and cool temperatures (i.e., animals that will emerge in the spring), and lighter forms when they develop under longer days and warmer conditions.

A number of species have now been examined for endocrine regulation of wing melanism polyphenism: the lycaenid *Lycaena phlaeas* (Endo and Kamata, 1985), the papilionids *P. xuthus* (Endo and Funatsu, 1985; Endo *et al.*, 1985) and *P. glaucus* (Koch *et al.*, 2000), and the nymphalids *Polygonia c-aureum* (Endo, 1984; Endo *et al.*, 1988; Fukuda and Endo, 1966), *Araschnia levana* (Koch and Bückmann, 1985, 1987), and *Junonia (Precis) coenia* (Rountree and Nijhout, 1995).

Although these species represent three families, and they differ in the direction of the polyphenism (i.e., which morph is induced under each set of conditions) and in whether or not the polyphenism is tied with facultative induction of diapause, the underlying physiological mechanisms show many similarities. All of these polyphenisms respond to both photoperiod and temperature, and although each factor can influence wing pattern expression independently, the effects of these environmental variables are additive in all cases. All of these polyphenisms involve critical periods of hormone sensitivity that occur early in the pupal period, and all but one involve the relative timing of the rise in ecdysteroids: when ecdysteroid levels are low during the critical period (i.e., when the pupal peak in ecdysteroid levels starts *after* the polyphenism sensitive period), then the default wing morph develops (e.g., *L. phlaeas*: Endo and Kamata, 1985; *A. levana*: Koch and Bückmann, 1987; *P. coenia*: Rountree and Nijhout, 1995; and probably *P. c-aureum*: Endo *et al.*, 1988; see also Rountree and Nijhout, 1995). When the ecdysteroid pulse is induced to start earlier, then high levels of ecdysone are present during the critical period, and animals switch to production of the alternate morph (**Figure 7**).

The link between external stimulation (e.g., exposure to temperature) and the timing of ecdysteroid secretion involves the brain, and secretion of neurohormones — either PTTH (presumed for *A. levana*, Koch and Bückmann, 1987 and *P. coenia*, Rountree and Nijhout, 1995) or another neurohormone called summer morph producing hormone (SMPH; Endo and Funatsu, 1985; Endo and Kamata, 1985; Fukuda and Endo, 1966; Tanaka *et al.*, 2009), which has structural similarities to both bombyxin and small PTTH (Masaki *et al.*, 1988; Rountree and Nijhout, 1995). Extirpation of brains in animals prevented release of these neurohormones resulting in complete production of the default morph (Endo and Funatsu, 1985; Endo and Kamata, 1985; Endo *et al.*, 1988; Fukuda and Endo, 1966; Rountree and Nijhout, 1995). However, injection of ecdysteroids as these brainless animals pupate (i.e., prior to the critical period) can restore the polyphenism and switch animals to the alternate form (Endo and Kamata, 1985; Koch and Bückmann, 1987; Rountree and Nijhout, 1995).

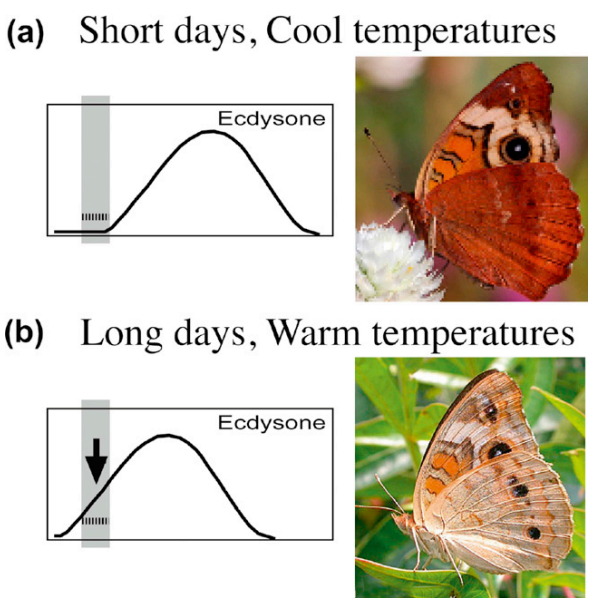

Figure 7 Seasonal wing color polyphenism in butterflies, as exemplified in *Junonia (Precis) coenia* represents the outcome of an interaction between allelic differences (genetic polymorphism for wing color) and environmental conditions. (a) Larvae exposed to a short-day photoperiod and low temperature regime secrete low levels of a small-PTTH-like neurohormone at the onset of the pupal phase. Consequently, the pupal ecdysteroid peak builds up late and reaches threshold levels only after the critical period (gray bar). This permits the expression of dark pigments in the developing wings which are, thus, adapted for higher solar absorption. (b) In contrast, exposure of larvae to a long-day photoperiod and high temperatures leads to an early and enhanced PTTH release, and consequently, above-threshold ecdysteroid levels during the critical period. These conditions favor the expression of the light colored, alternative wing phenotype. Based on data by *Masaki et al.* (1988), *Rountree and Nijhout* (1995), and other references cited in the text.

These results suggest the following model for light/dark polyphenism in butterflies (summarized for *P. coenia* in **Figure 7**): environmental stimuli affect the timing of ecdysteroid secretion indirectly via neurohormone secretion by the brain. Larvae exposed to warm temperatures and long days produce high levels of neurohormone. High neurohormone levels stimulate early secretion of ecdyseroids, and early secretion of ecdysteroids switches animals to production of the alternate (generally the lighter) morph.

11.3.1.2.2. Presence/absence of eyespots Some butterfly species exhibit seasonal polyphenism for pattern elements, in addition to, or instead of, seasonal variation in overall levels of melanization (Brakefield and Larsen, 1984; Brakefield and Reitsma, 1991; Condamin, 1973; Roskam and Brakefield, 1999; Shapiro, 1976). A striking example occurs in the satyrid *Bicyclus anynana,* which exhibits a wet versus dry season polyphenism for presence (and size) of eyespots (Brakefield *et al.*, 1998; Brakefield and Reitsma, 1991; Kooi *et al.*, 1996; Windig *et al.*, 1994). Wet season animals express pronounced ventral forewing eyespots that are reduced or absent in dry season animals.

Ventral eyespots are thought to aid animals in surviving attacks by avian predators because they misdirect the aim of the predator (Brakefield and Larsen, 1984; Windig *et al.*, 1994; Wourms and Wassermann, 1985). Wet season animals are active (feeding, mating, and ovipositing), and are regularly exposed to avian predators, and it is presumed that these animals survive better with pronounced eyespots. Dry season animals are almost completely dormant, and escape predators through crypsis. These animals achieve better crypsis if they do not produce conspicuous markings, such as eyespots (Brakefield and Larsen, 1984; Brakefield and Reitsma, 1991; Windig *et al.*, 1994).

Eyespot polyphenism in Southern Hemisphere populations of *B. anynana* results from larval sensitivity to temperature (Brakefield and Mazotta, 1995; Brakefield and Reitsma, 1991; Kooi and Brakefield, 1999; Kooi *et al.*, 1994; Windig *et al.*, 1994). Late-stage larvae exposed to temperatures above 23°C produce the "wet season" pattern with large eyespots. Larvae exposed to temperatures below 19°C produce the "dry season" form with tiny or no eyespots (Brakefield *et al.*, 1998; Brakefield and Mazotta, 1995). Although natural populations of these butterflies are dimorphic, this developmental mechanism (like the light/dark polyphenism described in the previous section, incidentally) does *not* seem to involve a threshold: intermediate temperatures lead to intermediate eyespot sizes, and animals reared in the laboratory can be induced to produce a continuous range of wing patterns (Windig *et al.*, 1994). Instead, dimorphism appears to arise from distinct seasonal temperature regimes encountered by sequential generations of larvae in the wild (Oostra *et al.*, 2010; Windig *et al.*, 1994).

Temperature-induced variation in eyespot size appears to be mediated by the timing of the pupal pulse of ecdysteroids (Brakefield *et al.*, 1998; Koch *et al.*, 1996; Oostra *et al.*, 2010; Zijlstra *et al.*, 2004). Just as with the light/dark polyphenism in *A. levana* and *P. coenia*, animals have a hormone-sensitive period during the first few days of the pupal period. The default developmental pathway appears to be a pupal pulse of ecdysteroids that starts *after* this critical period. When ecdysteroid levels are low during the sensitive period, no eyespots form (the dry season form). Wet season (warm-temperature) animals have an earlier pulse of ecdysteroids that precedes the sensitive period, and presence of ecdysone during this period results in the production of large eyespots (Brakefield *et al.*, 1998; Koch *et al.*, 1996; Oostra *et al.*, 2010).

Genetic strains of *B. anynana* that have been selected for continuous expression of the no-eyespot morph have late pulses of ecdysteroids identical to those of the cool-temperature-induced dry season animals (Brakefield *et al.*, 1998; Koch *et al.*, 1996). However, these genetically eyespot-less animals can be induced to produce eyespots by injections of ecdysone prior to the sensitive period (Koch *et al.*, 1996). These results all point to a mechanism where polyphenic expression of butterfly eyespots results from a coupling of larval exposure to temperature with variation in the timing of secretion of ecdysone during the first 2 days of the pupal period (**Figure 8**), a model confirmed recently by ecdysteroid titer measures across a range of developmental temperatures (Oostra *et al.*, 2010).

Butterfly eyespots have served as a model system for characterizing the developmental control of pattern formation (Beldade and Brakefield, 2002; Brunetti *et al.*, 2001; French, 1997; French and Brakefield, 1995; Kühn and von Engelhardt, 1933; McMillan *et al.*, 2002; Nijhout, 1985, 1986, 1991; Wittkopp and Beldade, 2008), and recent genetic experiments provide exciting glimpses into the patterns of gene expression that underlie eyespot formation in general, and temperature-sensitive modulation of eyespot size in particular.

Butterfly eyespots result from a patterning mechanism that takes place in the wings of late larval and early stage pupae (Beldade and Brakefield, 2002; Brunetti *et al.*, 2001; French, 1997; French and Brakefield, 1995; Nijhout, 1985, 1986, 1991). Briefly, the eyespot consists of a series of concentric pigmented rings around an organizing center, or focus. Cells in the focus of an eyespot are critical for normal formation of the eyespot; if these cells are ablated at the end of the larval period, eyespots fail to form (Nijhout, 1980, 1991; Nijhout and Grunert, 1988). Likewise, if foci cells are transplanted, they can induce ectopic eyespots in other parts of the developing wing (Brakefield *et al.*, 1996; French and Brakefield,

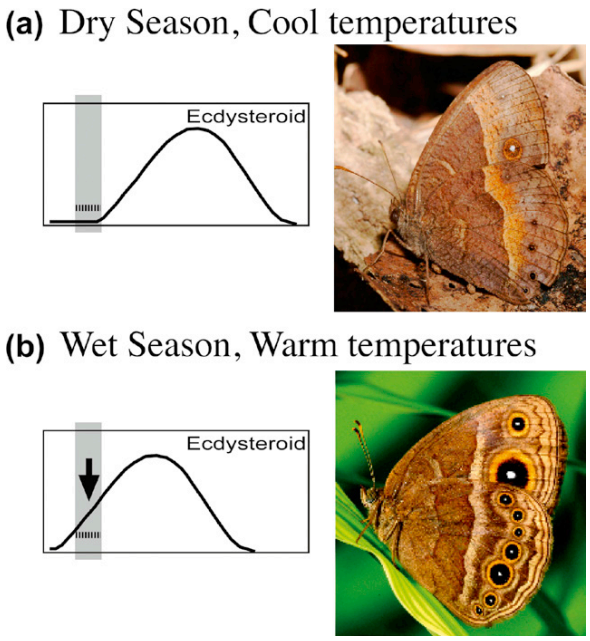

Figure 8 Eyespot size polyphenism as exemplified in the tropical butterfly *Bicyclus anynana*. (a) The less active dry-season (low temperature) morph exhibits small eyespots on the ventral side of the forewings as a result of a low ecdysteroid titer during the critical period (shaded bar) at the onset of pupal development. (b) In contrast, the forewings of the wet-season (high temperature) morph develop large eyespots in response to an above-threshold ecdysteroid titer during this critical period. In the wing discs of last instar larvae, eyespot focal cells express the Distal-less protein. The earlier pupal ecdysteroid peak causes enhanced synthesis of dark pigment from this focal center and, consequently, generates large eyespots in the wet-season morph. Based on data and models by *Brakefield and Reitsma* (1991), *Kooi et al.* (1994), *Brakefield et al.* (1996, 1998), *Koch et al.* (1996, 2003), *Brunetti et al.* (2001), and *Beldade and Brakefield* (2002). For further references, see the text. Photos courtesy William Piel and Antonia Monteiro.

1995; Nijhout, 1991). It appears that foci establish the eyespot by secreting a diffusible chemical morphogen into the surrounding epidermis, and this signal is interpreted by the surrounding cells in a way that affects the color of the pigment synthesized; gradient contours in the diffusible substance interact with the relative sensitivities of the surrounding cells to generate the concentric rings of the eyespot (Dilao and Sainhas, 2004; Evans and Marcus, 2006).

Although the identity of the morphogen released from eyespot foci is not yet known, recent genetic studies using antibodies for *Drosophila* patterning genes have identified several signaling molecules and transcription factors expressed in the appropriate areas and at the appropriate times (Beldade and Brakefield, 2002; Brakefield *et al.*, 1996; Brunetti *et al.*, 2001; Carlin *et al.*, 1994; Keys *et al.*, 1999; Weatherbee *et al.*, 1999). For example, *distal-less*, *engrailed* and *spalt* all have circular regions of expression that correspond with the position of eyespots (Beldade *et al.*, 2002; Brakefield *et al.*, 1996; Brunetti *et al.*, 2001; Keys *et al.*, 1999; Monteiro *et al.*, 2006) and their expression occurs at the time of eyespot pattern formation (late larval and early pupal period). All three genes are expressed together in the organizing focus of the eyespot, but they are expressed separately in the surrounding color rings, lending credence to the idea that they may in some way contribute to the patterning of the rings themselves (Beldade and Brakefield, 2002; Brunetti *et al.*, 2001).

Brakefield and colleagues have taken the next step by linking expression patterns of these genes — *distal-less* in particular — with quantitative variation in eyespot size. Genetic lines selected for large and small eyespots differ in the relative size of the eyespot focus, as measured by the region of *distal-less* expression (Beldade and Brakefield, 2002; Beldade *et al.*, 2002; Monteiro *et al.*, 1994, 1997), and these authors then showed that genetic variants of *distal-less* expression co-segregate with inter-individual variation in eyespot size.

The *wingless* gene is also expressed at appropriate times and locations in eyespot centers (Monteiro *et al.*, 2006). However, it is not yet clear how these gene products interact with each other during eyespot formation (Evans and Marcus, 2006; Saenko *et al.*, 2007) or how they regulate pigment synthesis (Koch *et al.*, 2000), and it is already apparent that different eyespots can utilize different combinations of these patterning elements (e.g., eyespots in Pieris spp. are patterned by different genes than eyespots in Nymphalid spp.; Monteiro *et al.*, 2006). Extensive genomic tools have now been developed for *Bicyclus*, including high-density genetic arrays, dense genetic maps, and genetic transformation techniques (Beldade *et al.*, 2006, 2008; Marcus *et al.*, 2004), which will facilitate further investigation of these patterning mechanisms.

Combined, these studies suggest an explicit model for the developmental basis of variation in eyespot size. According to Beldade and Brakefield (2002), differences in the relative amount of growth of the organizing focus lead to differences in the size of the focus at the time of pattern formation. This translates into the strength of the signal emitted from this organizing center and results in relatively larger or smaller eyespot diameters. Plasticity in the expression of eyespot size could then result from a coupling of the amount of growth of the organizing focus with the amount of ecdysteroid present during the patterning period (Koch *et al.*, 2003). Wet season (higher temperature) animals would have early rises in pupal ecdysteroid levels, stimulating growth of large eyespot foci, resulting in correspondingly large adult eyespots. Dry season (lower temperature) animals would have later rises in pupal ecdysteroid levels, less hormone present during the sensitive period, less growth of the eyespot foci, and relatively smaller final eyespots (**Figure 8**).

Interestingly, not all eyespots are plastic — even within the same individual. In *B. anynana* ventral forewing eyespots are exquisitely sensitive to the larval thermal environment, but dorsal eyespots are not. Dorsal eyespots are expressed in all individuals and display no detectable plasticity. Brakefield *et al.* (1998) proposed that this difference in the environmental sensitivity of expression of dorsal and ventral eyespots results from the presence of ecdysteroid receptors in the foci of ventral eyespots, and the absence of these same receptors in the focal cells of the dorsal eyespots. Thus, patterns of ecdysone receptor expression may underlie the coupling of an adult wing pattern element expression (eyespots) with seasonally variable components of the larval environment (temperature).

11.3.2. Coleoptera — Male Dimorphism for Weaponry

Males of many species face intense competition from rival males over access to reproduction (Andersson, 1994; Darwin, 1871; Thornhill and Alcock, 1983). In these species, it is not uncommon for dominant and subordinate individuals to adopt different behavioral tactics: dominant (generally large) males fight to guard territories frequented by females or display or sing to attract females, while subordinate (generally smaller) males of these same species adopt less aggressive alternative tactics, such as sneaking up to or mimicking females (Austad, 1984; Darwin, 1871; Dominey, 1984; Gross, 1996; Iguchi, 1998; Oliveira *et al.*, 2008; Shuster, 2002).

In the most extreme cases, large and small males differ in morphology as well as reproductive behavior. Rarely, these alternative male "morphs" result from allelic differences among the male types (Lank *et al.*, 1995; Ryan *et al.*, 1990; Shuster, 1989; Zimmerer and Kallmann, 1989). Instead, the majority of male dimorphisms appear to result from polyphenic developmental processes that switch among alternative phenotypic possibilities depending on larval growth, and/or the resulting body size attained by each individual (Clark, 1997; Eberhard, 1982; Goldsmith, 1985, 1987; Kukuk, 1966; Rasmussen, 1994; Tomkins, 1999). Males encountering favorable conditions grow large and produce one morphology, while genetically similar (e.g., sibling) individuals encountering poor conditions remain small and produce an alternative morphology. These male-dimorphic polyphenisms are characterized by a relatively abrupt switch between morphs that corresponds with a critical, or threshold, body size (Cook, 1987; Danforth, 1991; Diakonov, 1925; Eberhard and Gutierrez, 1991; Emlen *et al.*, 2005a; Iguchi, 1998; Kawano, 1995; Kukuk, 1966; Rasmussen, 1994; Tomkins and Simmons, 1996).

Only one male-dimorphism that we are aware of has been characterized physiologically — the alternative horn morphologies of the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *Onthophagus taurus* is a European species that has been introduced into both Australia and the United States, where it is now an abundant inhabitant of horse and cow manure (Fincher and Woodruff, 1975; Tyndale-Biscoe, 1996).

Beetles fly into fresh manure pads and excavate tunnels into the soil below. Females dig the primary tunnels and spend a period of days pulling fragments of dung to the ends of these tunnels, where they pack them into oval masses called "brood balls" (Emlen, 1997; Fabre, 1899; Hallffter and Edmonds, 1982; Moczek and Emlen, 2000). A single egg is laid inside each brood ball, and larvae complete their development in isolation within these buried balls of dung (Emlen and Nijhout, 1999, 2001; Fabre, 1899; Main, 1922).

Male behavior revolves around methods of gaining entry to tunnels containing females. Large males fight to guard tunnel entrances, while smaller males sneak into these tunnels on the sly (Emlen, 1997; Moczek and Emlen, 2000). Large males produce a pair of long, curved horns that aid them in contests over tunnel occupancy (Moczek and Emlen, 1999). Smaller males dispense with horn production altogether.

Both overall body size and male horn length are sensitive to the larval nutritional environment (Emlen, 1994; Hunt and Simmons, 1997; Moczek and Emlen, 1999). Specifically, the amount and quality of larval food predictably influence the final body size and horn lengths of males (Emlen, 1994; Hunt and Simmons, 1997; Moczek and Emlen, 1999). Controlled breeding experiments, artificial selection experiments, population comparisons, and "common garden" experiments all suggest that horn expression is regulated by a threshold mechanism, and that natural populations contain measurable levels of additive genetic variation for the body size threshold, that is, the size associated with the switch between horned and hornless morphologies (Emlen, 1996; Moczek *et al.*, 2002). Somehow, then, the relative amount of growth of the horns must be influenced by the overall growth, or body size, attained by each animal. Specifically, this polyphenism appears to involve a reprogramming of animals that fall beneath a genetically mediated threshold body size, so that in these animals, growth of the horns is reduced (Emlen *et al.*, 2005a, 2006; Moczek, 2006, 2007).

Beetles pass through three larval instars before molting into a pupa (Emlen and Nijhout, 1999; Main, 1922). Larvae feed on dung supplies that are provided in the brood ball, and gain weight steadily. When these food supplies are depleted, a stereotyped series of events is commenced and this ultimately results in the metamorphic molt from larva to pupa (Emlen and Nijhout, 1999). The cessation of feeding appears to trigger a rapid drop in JH titers analogous to the drop that occurs with attainment of a critical size for metamorphosis in *Manduca sexta* (Nijhout, 1994). As animals stop feeding, they begin to

purge their gut, they begin forming a mud/fecal shell that will protect them as pupae, and the imaginal structures (legs, wings, genitalia, horns) begin to grow.

A combination of hormone-application perturbation experiments (JH) and radioimmunoassays of hormone titer profiles (ecdysteroids) revealed two critical periods relevant to this developmental polyphenism. Perturbation of hormone levels during either of these sensitive periods influences the expression of male horns, although the effects of hormone application at these two times are distinct (Emlen *et al.*, 2005b; Emlen and Nijhout, 1999, 2001). The first of these critical periods occurs at the end of the feeding period, before initiation of the metamorphic molt. Animals at this time have attained their largest sizes, and larval weight during this period exactly predicts patterns of male horn expression. Male larvae with sustained weights equal to or heavier than 0.12 g end up producing horns, whereas male larvae not sustaining this critical weight do not produce horns (Emlen and Nijhout, 2001). Furthermore, male larvae with weights beneath this critical size have a small pulse of ecdysteroids that is not present in larger males. These results suggest that body size is assessed at this time, and that animals beneath a critical larval weight are "reprogrammed" by a morph-specific pulse of ecdysteroids (also see Moczek, 2006). Levels of JH appear to be involved in this size-assessment process. Topical application of the JH analog methoprene during this critical period raised the threshold body size associated with horn expression, so that methoprene-treated animals needed to attain a larger body size (heavier larval weight) for horn production than acetone-treated control animals (Emlen and Nijhout, 2001).

A second critical period occurs after the metamorphic molt has been initiated, as animals purge their guts and enter the pre-pupa period. This is the period of horn growth. All of the imaginal structures, including the horns, are growing rapidly at this time, and all animals have very high levels of ecdysteroids irrespective of sex or morph (Emlen and Nijhout, 2001). Here again levels of JH appear correlated with body size, and perturbation of levels of JH influence the relative amount of growth of imaginal structures. In this case, augmentation of levels of JH (by topical application of methoprene) caused animals to produce disproportionately large horns. Specifically, it caused small, typically hornless males to produce horns (Emlen and Nijhout, 1999; Moczek *et al.*, 2002).

In summary, experiments suggest that there are at least two periods during larval development when horn expression is sensitive to endocrine events. A first critical period occurs as animals attain their largest body sizes, and animals not attaining (or sustaining) a threshold size get reprogrammed by a small, morph-specific pulse of ecdysteroids at this time. Ecdysteroids affect patterns of gene transcription and are known to reprogram the developmental fates of specific structures (e.g., epidermis, imaginal discs; reviewed in Nijhout, 1994). In this case, a pulse of ecdysteroids may reprogram the relative sensitivity of cells destined to become the horns, so that their growth is inhibited during the pre-pupal period when all of the imaginal structures grow to their full sizes.

Advances have now been made in understanding the genetic mechanisms underlying horn growth, such as how these structures arose and how they were subsequently modified in form as species in this genus diversified. The origin of beetle horns appears to have entailed the co-option of portions of traditional appendage patterning, in particular components of the network of genes responsible for patterning the proximo-distal axis (Emlen *et al.*, 2006, 2007; Moczek and Nagy, 2005; Moczek and Rose, 2009; Moczek *et al.*, 2006). Yet beetle horns are fantastically diverse, exhibiting stunning variety in shapes, sizes and types (e.g., head versus thorax). A phylogenetic analysis of 48 species of the genus *Onthophagus* revealed multiple origins of horns as well as losses (Emlen *et al.*, 2005a,b). These structures clearly have been gained and lost repeatedly in the history of this genus, a pattern reflected in emerging studies of horn development: it is already evident that horns in different *Onthophagus* species — *and* even different horn types in the same species — utilize different subsets of the appendage patterning network in the regulation of their growth (Moczek and Nagy, 2005; Moczek and Rose, 2009; Moczek *et al.*, 2006).

Polyphenic regulation of horn expression (horn dimorphism) also has been gained and lost repeatedly in these beetles, so that closely related species may differ in whether their horns are dimorphic and in the nature of their dimorphism (Emlen *et al.*, 2005b). It has even been suggested that multiple threshold mechanisms may operate *simultaneously on the same horn* to yield populations with three distinct male types (facultative male trimorphism; Rowland and Emlen, 2009). This pattern of rapid and prolific evolution of polyphenic mechanisms is reflected in preliminary studies of their underlying developmental regulation. There clearly are several different ways that horn growth can be truncated in small males (and females). The most obvious entails the prevention of proliferation of horns in the first place — a process that occurs in the head horns of *O. taurus*, and to some extent, in the thoracic horns of *O. nigriventris* (Emlen *et al.*, 2006). Preliminary studies of transcription of the *O. nigriventris* insulin receptor gene (InR) suggest that localized interruption of insulin signaling in the horn tissues may contribute to the truncation of horn cell proliferation in small males and females (Emlen *et al.*, 2006; Lavine *et al.*, unpublished results).

It is also possible for dimorphism to arise after this period of growth during the pupal period when epidermal tissues are remodeled prior to the formation of the adult cuticle (**Figure 9**). Moczek showed that significant remodeling of beetle horns can occur during this period,

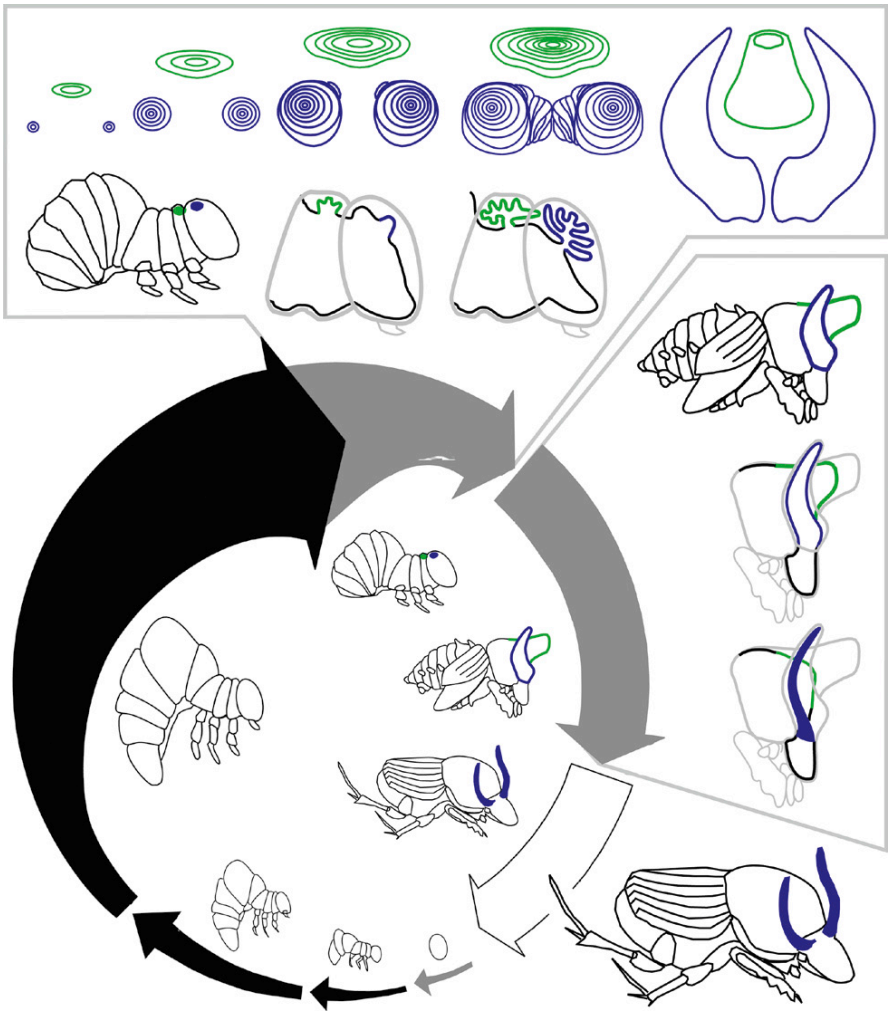

Figure 9 Polyphenic regulation of beetle horns can arise during either of two stages of horn development. Horns grow during a brief, non-feeding, period at the end of the third (final) larval instar and the pre-pupal period, and this process can be truncated to produce hornless individuals. After animals molt into pupae, the epidermal cells begin to produce the adult cuticle. At this time, significant remodeling of horn tissues can occur, probably through localized patterns of cell death. Dimorphism in horn expression can be generated or erased, depending on the amount of horn tissue loss that occurs in large males, small males, and females, respectively. Life cycle for *Onthophagus taurus* shown with arrow size roughly corresponding to animal size, and black arrows indicating feeding periods. In this species, thoracic horns (green) are grown during the pre-pupal period and removed completely in all individuals during the pupal period. Head horns (blue) proliferate in large males, but not in small males or females, and undergo only minor remodeling during the pupal period. Profile drawings modified from *Moczek* (2006). Reproduced with permission from *Emlen et al.* (2007).

and that this could generate dimorphism in horns that initially grow in all individuals of both sexes, which are subsequently reabsorbed in small males and females (Moczek, 2007; Wasik *et al.*, 2010). From this it is abundantly clear that the regulatory mechanisms are diverse, with either or both of these two processes occurring in each horn type; across different horn types (even within the same species), the mechanisms responsible for facultative horn loss vary. It appears that endocrine events near the end of the larval feeding period specify either a horned or a hornless trajectory for subsequent development, and that these regulatory events are implemented either during the pre-pupal period, through differential amounts of horn tissue proliferation, or during the pupal period, through differential amounts of horn tissue reabsorption, to generate horned and hornless adult beetle morphologies.

Genomic tools are being developed for the dung beetle species *O. taurus* (Snell-Rood *et al.*, 2010) and *O. nigriventris* (Snell-Rood *et al.*, 2010; Warren, Lavine and Emlen unpublished results) as well as for the rhinoceros beetle *Trypoxylus dichotomus* (Warren, Lavine, and Emlen unpublished results), and these should permit the identification of suites of genes whose expression differs between horned and hornless morphs. Just this year, Snell-Rood *et al.* (2010) found that expression profiles for horn

tissues in hornless males of both *O. taurus* and *O. nigriventris* were more similar to the profiles of females than they were to those of larger, horned males (**Figure 10**). These exciting results suggest that the magnitude of developmental reprogramming involved in male dimorphism rivals that which occurs between the sexes. They also are consistent with the observation that small, hornless males appear to have converged on a body morphology similar to that of the females.

11.3.3. Hymenoptera — Caste Polyphenism and Division of Labor

Except for termite caste polyphenism, most of the examples discussed so far can easily be accommodated within the framework of classic Darwinian fitness concepts, wherein alternative phenotypes prove to be intimately related to alternative reproductive strategies. In principle, each of the selective environments encountered by insects

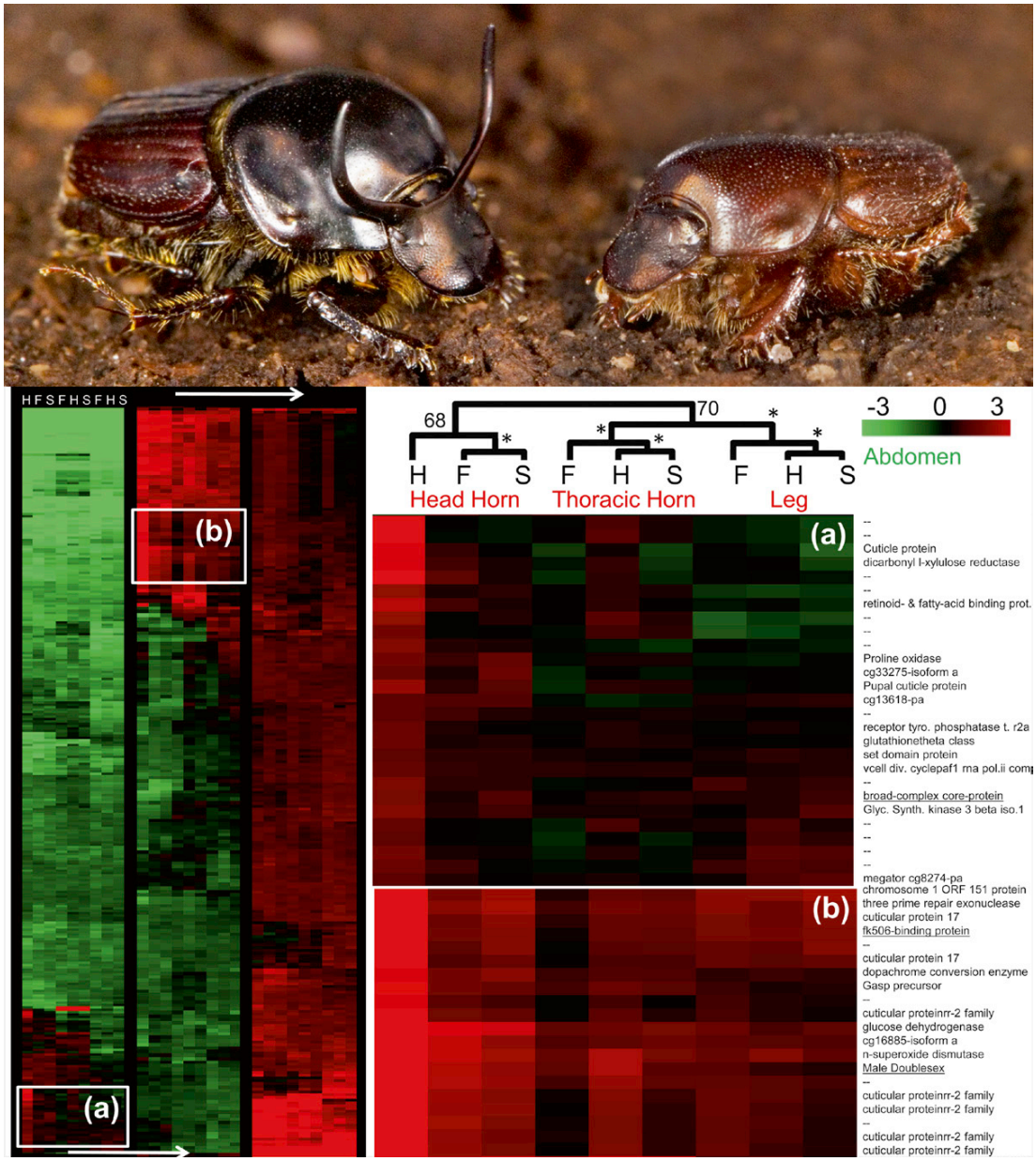

Figure 10 Polyphenic expression of horns in the dung beetle *Onthophagus taurus*. (a) Depending on the larval nutritional environment, males either develop into large adults with a pair of long, curved head horns (right), or into smaller adults with only rudimentary horns (left). (Photo courtesy of D. Emlen.) (b) Differences in patterns of horn growth are reflected by differential transcription of genes. Expression profiles for head tissues of small, hornless males (S) more closely resemble comparable tissues of females (F), than they do those of large, horned males (H). Photos D. Emlen; bottom panel reproduced with permission from *Snell-Rood et al.* (2010).

(e.g., spring vs. summer) favors a different optimal phenotype, and individuals maximize their reproductive success by expressing the appropriate morphology in the appropriate circumstance.

Caste polyphenisms, in contrast, are associated with a severe reduction in fertility of the subdominant (worker) caste. Workers in most social insect colonies never reproduce. Social insect societies with their complex caste systems, thus, do not obey the rules of classical Darwinian theory, a fact which has clearly and succinctly been stated by Darwin (1859). The dilemma of inserting social insect castes into a fully acceptable Darwinian framework was only resolved by the introduction of the theory of inclusive fitness and kin selection (Hamilton, 1964). Since then, a great deal of work has centered on ultimate (evolutionary) explanations of why individuals should refrain from reproducing. Numerous studies also address proximate (ontogenetic) mechanisms underlying developmental trajectories into reproductive individuals and non- or less-reproductive helpers (workers). A major issue that remains to be elucidated is the evolution of these proximate mechanisms, that is, the evolution of developmental pathways that generate the distinct queen/worker and also the soldier phenotypes. Only if it becomes possible to map these physiological mechanisms onto a phylogenetic framework may a unifying picture of caste evolution emerge that satisfies both proximate and ultimate explanations.

The diversity in caste syndromes and the manifold stimuli that trigger caste differentiation remain a challenge to any unifying view of developmental regulation. In part, the root of this problem lies in the conceptual framework of the caste. In the most general sense, the term caste is used to describe *functional* roles in reproduction and division of labor, such as the performance of different tasks by different members of an insect society, independent of whether these roles are a reflection of behavioral differences only, or whether there are also differences in morphological phenotype. In a more restricted sense, a caste is seen as a manifestation of pre-imaginal developmental diversification resulting in *morphologically distinct phenotypes*, which are then ever more prone to perform distinct functions in the division of labor. Distinct caste phenotypes are a hallmark of the highly eusocial insect societies, and in a discussion of insect polyphenism one might tend to concentrate on this latter aspect. Yet, in terms of evolutionary explanations, that is, how such morphologically distinct phenotypes may have emerged from modifications in developmental physiologies and gene expression, it is informative to start with incipient social systems that arise from individual differences in reproductive potential. In the subsequent sections, we will try to discern these two aspects of caste and the underlying endocrine mechanisms that govern behavioral and morphological diversification.

A first and major distinction in social insect organization and caste systems sets apart the hemimetabolous termites from the holometabolous Hymenoptera (wasps, bees, and ants). As previously outlined, termites are diplo-diploid hemimetabolans that descended from presocial cockroaches, and caste development in termites is essentially a problem of how endocrine regulation of post-embryonic development maintains immatures as a worker caste while permitting terminal differentiation of a soldier and a reproductive caste. Sociality in the Hymenoptera, in contrast, is built on asymmetries of genetic relationships generated by the haplo-diploid system of sex determination (Hamilton, 1964), and arose several times independently. Multiple evolutionary origins of sociality make hymenopterans, and in particular wasps and bees, useful objects to study the environmental, genetic, and endocrine background that set the stage for the development of the exclusively female caste phenotypes, including the primary ones — the queens and workers. Ants, in contrast, are all highly social, and thus are less ideally suited for comparative studies with such a focus. They are most valuable, however, when it comes to testing hypotheses on the evolution of multiple sterile female caste phenotypes, in particular, worker versus soldier development. It is important to emphasize at this point, that the soldier caste in ants is a highly derived phenotype that makes its appearance only in a few genera, in contrast to the soldier caste in termites, which is one of the essential features of termite sociality.

In general terms, holometabolous caste phenotypes can be seen as a progressive fixation of roles, first in reproduction, and secondarily in the specialization to specific tasks related to colony maintenance (Wilson, 1971). Evolutionarily, the monopolization of colony reproduction by a queen caste is a conflict-ridden situation that starts out with asymmetries in reproductive potential, either of females that are cofoundresses of a nest, or results from the repression of the reproductive potential of offspring daughters by the mother who persists in the nest. In the first case reproductive dominance should become established through a series of dominance interactions between adult and potentially reproductive females, whereas in the latter case, which requires an overlap of generations, the dominant egg-laying mother may repress reproductive activity in her daughters by manipulating a pre-imaginal caste bias.

11.3.3.1. Wasps As stated by West-Eberhard (1996): "wasps are a microcosm for the study of development and evolution" of insect sociality, and they serve as a logical baseline for endocrine studies of caste polyphenism. All social Hymenoptera evolved from wasps (Wilson, 1971), and most levels of sociality can be found within extant wasp taxa. Three of the subfamilies in the Vespidae, the Stenogastrinae, Polistinae, and Vespinae contain social species (Carpenter, 1991; Pickett and Carpenter, 2010) with the Polistinae being of special interest since they

represent practically the entire range of social evolution. Many of the Old World Polistinae are open-nesting wasps, and colonies can be founded by one or more mated females. In such incipient colonies, a dominance hierarchy is gradually established by aggressive interactions among adult females (Sledge *et al.*, 2001). Thereafter, the dominant female signals her status to the subordinate females (Sledge *et al.*, 2001), and whenever she encounters an egg laid by another female she removes it. In such a system, foregoing reproduction with the perspective to eventually substitute the dominant female and taking over the nest has a relatively high value in the pay-off matrix, since survival chances of individual nest-founding females generally are quite low.

The endocrine basis of this reproductive dominance has initially been investigated in *Polistes dominulus* (formerly *P. gallicus*). These studies revealed a synergistic interaction between JH synthesis, ecdysteroid titer, and ovarian activity. Dominant females have higher CA activity and consequently a higher JH titer than subordinate females. In addition, the higher ovarian activity in dominant females also correlates with an elevated ecdysteroid titer. JH and/or ecdysteroid treatment of subordinate females resulted in a rise in their social rank (Barth *et al.*, 1975; Röseler *et al.*, 1984, 1985; Strambi, 1990). Such an interaction in hormonal control of physiology and behavior is a general trait in insect reproduction, and should be expected also in solitary or incipiently social Hymenoptera. These studies, however, also pointed out an important gap in our comprehension of reproductive dominance, that is, the distinction between the presence of an activated ovary and high social rank (Sledge *et al.*, 2001). Even when ovariectomized, some *Polistes* foundress females continued to maintain their dominant status (Röseler *et al.*, 1985). Generally, these were females with large CA. A large CA volume (and thus presumed high CA activity), however, was not consistently linked to dominance, since in other cases formerly subordinate females that became dominant over ovariectomized foundresses did not have larger CA. Recent results shed light on the multifactorial interplay in conflict and its resolution among *P. dominulus* females. Information on fighting ability is conveyed by facial signals that allow individual identification (Tibbetts and Huang, 2010), whereas cuticular hydrocarbon profiles indicate reproductive status (Izzo *et al.*, 2010). This multisignal situation is also reflected in JH titers. After queen removal, the JH titers are upregulated in workers as a response to this social stress and aggression-rich situation, whereas there is no relation between JH titer and aggression in queenright colonies (Tibbetts and Huang, 2010). Furthermore, JH appears to play a role in division of labor among workers of stable colonies, advancing the onset of foraging activity in the lifetime of individual wasps (Tibbetts and Izzo, 2009).

With increasing social complexity the choice between alternative reproductive strategies evidently becomes restricted, and in parallel, morphological caste differences become implemented and increase in degree. This is nicely illustrated in the Ropalidiini, where a gradual shift toward a preimaginal caste bias has been convincingly demonstrated (Gadagkar *et al.*, 1988), together with an age-dependent pattern of task performance (temporal or age polyethism) in adult workers (Naug and Gadagkar, 1998).

In the Neotropical, swarm-founding Epiponini, morphological caste phenotypes are clearly expressed ranging from subtle differences between the extremes in a unimodal body size range, to clearly dimorphic castes resulting from non-isometric growth in the preimaginal phase. In the latter, queens are not necessarily always the largest individuals. Allometric analyses suggest size-independent allometries in many of these cases as seen by pre-imaginal caste determination marked by a resetting of growth parameters in critical phases of development (Jeanne *et al.*, 1995; Keeping, 2002; Noll *et al.*, 1997; O'Donnell, 1998). Furthermore, these analyses showed that the caste system in most genera of Epiponini appears to have originated from an ancestral lineage that did not have a queen caste (Noll and Wenzel, 2008).

Neither the Ropalidiini nor the Epiponini have been investigated regarding endocrine system functions in caste development, and only in two species has hormonal control in division of labor among the adult wasps been addressed. The results are of interest as they indicate a split in reaction patterns. In the primitively social *Ropalidia marginata*, an elevated JH titer stimulated egg development (Agrahari and Gadagkar, 2003), whereas in the socially more advanced *Polybia occidentalis*, treatment with the JH analog methoprene accelerated behavioral development in workers (O'Donnell and Jeanne, 1993), similar to what is observed in honey bees.

As in other insects, genomic studies are also gaining momentum in wasps, where a large EST library for *P. metricus* furthered the establishment of a platform for high throughput gene expression analyses (Toth *et al.*, 2007). Brain gene expression of workers that were rearing brood is similar to that of foundresses (that also rear brood) and markedly differs from queens. Among the differentially expressed genes were several related to the insulin signaling pathway, inferring a strong connection between nutritional and reproductive regulation in *Polistes* castes (Toth *et al.*, 2007). A second link of interest in developmental regulation of caste comes from a candidate gene analysis and proteomic screen showing that prospective queen and worker larvae differ in the expression pattern of diapause-related genes (Hunt *et al.*, 2010). The potential to become a reproductive (gyne) among *Polistes* female offspring, which becomes established during larval development, thus appears to be contingent on a diapause-related gene

network. The latter represents a facultatively expressed trait in the life history of ancestral non-social wasps (Hunt *et al.*, 2007b).

11.3.3.2. Ants Like their hemimetabolan counterparts, the termites, ants are an apex in caste complexity, and thus, also a challenge to any unifying hypothesis on caste development and function. In this section we provide only a glimpse into the ant empire of behavioral and developmental diversity. Luckily, we can direct any reader interested in a more detailed presentation on ant biology to the masterpiece of scientific literature written by Hölldobler and Wilson (1990).

Prior to any further discussion it is important to emphasize that there are no solitary or primitively social ants. Possibly related to a major switch in foraging biology from flying to walking in search of prey, a mesozoic sphecoid wasp-like ancestor to ants appears to have been exposed to relaxed selective constraints on the aerodynamics of body shape, making possible a wide variation in worker ant morphologies. This resulted in an eminent wing polyphenism between queens and workers, which is evidenced already in the earliest ant fossils (Wilson, 1987). Many early studies on ant sociobiology capitalized on such variation in form, analyzing size allometries in relation to functions performed by colony members and in relation to phylogenetic patterns (reviewed in Hölldobler and Wilson, 1990; Wheeler, 1991; Wilson, 1971). Even though there is a wealth of data on complex trait allometries in ants, there still appears to be a lack of information linking these data to rules of *how* changes in shape are actually generated in development, and how these specific developmental pathways have evolved (Tschinkel, 1991; Tschinkel *et al.*, 2003).

11.3.3.2.1. The Queen/worker decision As in all highly social insects, ant queens differ markedly from workers. They are generally bigger, have a fully developed reproductive system, and have a well-developed flight apparatus for dispersal. Even though this queen/worker distinction is an ancestral character present in all ant species, we still have very limited knowledge as to how these caste differences arise ontogenetically. Many of the early studies on mechanisms governing queen production in ants were carried out on temperate climate species, which showed strong seasonal triggers for queen production (reviewed in Wheeler, 1986). In many species, only queens are produced from overwintered eggs or larvae, illustrating a requirement for a state of diapause in the development of queens.

For some ants, the critical period for the queen/worker decision has been studied by JH application experiments. For example, topical application of JH to larvae developing from queen-biased overwintered brood in *Myrmica rubra* increased the number of queens (Brian, 1974). In *Aphaenogaster senilis*, application of JH caused the appearance of queen-like workers (Ledoux, 1976), and this same pattern occurred in the Argentinian fire ant, *Solenopsis invicta* (Vinson and Robeau, 1974). In the latter species, metamorphosis was delayed significantly by the application of JH, and the "queen-like" appearance of workers was initially attributed to a simple increase in worker size (associated with the delay in metamorphosis, and extended period of larval feeding), rather than to a direct effect of JH on queen production (Wheeler, 1990).

Ecdysteroids also have been implicated in divergent queen/worker development. In *Plagiolepis pygmea*, worker-biased larvae have higher ecdysteroid titers during the last larval instar than queen-biased larvae (Suzzoni *et al.*, 1983).

In all examples cited so far caste development is dependent on environmental factors. Yet, there is now increasing evidence for a genetic basis to the determination of queen/worker polyphenism in at least some species of ants. Such genetic factors (allelic differences between queens and workers) were originally proposed for the slave-making ant *Harpagoxenus sublaevis* (Winter and Buschinger, 1986), but have recently also been demonstrated for a *Camponotus* (Fraser *et al.*, 2000), two *Pogonomyrmex* species (Julian *et al.*, 2002; Volny and Gordon, 2002), and the very peculiar sex/caste determination system in the little fire ant *Wasmannia auropunctata* (Foucaud *et al.*, 2010). A yet unresolved question is how the allelic combinations setting up the genetic basis for caste interact with the endocrine system.

Environmental triggers of ant caste development primarily involve pheromonal signals within the colony. Pheromonal regulation of ant reproduction has primarily been studied in *S. invicta*, which has completely sterile workers due to the lack of functional ovaries (Hölldobler and Wilson, 1990). Virgin *Solenopsis* queens normally leave to undertake a mating flight and thereafter shed their wings, activate their ovaries, and soon initiate egg laying. If prevented from flying, these gynes keep their wings and maintain their ovaries in an inactive state. Furthermore, gynes are prevented from maturing their ovaries as long as an egg-laying queen is present in the nest (Fletcher and Blum, 1981). This integrated behavioral and physiological response in *Solenopsis* virgin queens has led to the identification of a pheromone produced by the poison gland of the dominant queen, which inhibits precocious dealation and ovary activation in virgin queens as long as they are in the nest (Fletcher and Blum, 1983). The pheromone supposedly maintains high brain dopamine levels (Boulay *et al.*, 2001) that are thought to suppress CA activity (Burns *et al.*, 2002; Vargo and Laurel, 1994). Low CA activity in turn prevents vitellogenin uptake by the ovary. Vitellogenin synthesis appears to occur independent of JH (Vargo and Laurel, 1994), and the decision to initiate oogenesis or not seems to be taken by the activation

of receptor-mediated vitellogenin uptake. In *Camponotus festinatus*, such a regulation has also been shown to prevent ovary activation in queenless workers (Martinez and Wheeler, 1991). Another interesting endocrine regulatory mechanism was found for the harvester ant *Pogonomyrmex californicus*, where queens can either found a nest alone or join into a group of cofounding queens exhibiting division of labor, especially with respect to foraging decisions. In both cases, JH titers were found to be elevated during the foraging stage (Dolezal *et al.*, 2009), revealing a foraging bias elicited by this hormone similar to what is known for honey bee workers.

The species considered so far all belong to a large group of ants that do not exhibit a marked polyphenism within the worker caste. Instead, they all have a worker caste with a unimodal or only slightly bimodal size frequency distribution. In these species, the principle polyphenism is between queens (reproductives) and workers (non-reproductives). In contrast, the genus *Pheidole* is characterized by multiple polyphenisms, such as between queens and workers and also between different types of workers (including true soldiers). The genus *Pheidole* exhibits the most spectacular polyphenism known for social insect worker castes (see the next section). In this genus, the queen/worker polyphenism occurs much earlier in development than the worker/soldier switch to the extent that queen determination in *Pheidole* appears to occur in embryonic development by maternal factors, primarily hormones deposited in the egg during oogenesis. Queens that laid worker-biased eggs had higher ecdysteroid levels than those laying queen-biased eggs, and, correspondingly, worker-biased eggs also exhibited a higher ecdysteroid content (Suzzoni *et al.*, 1980). Furthermore, JH application to eggs increased the proportion of females developing into queens (Passera and Suzzoni, 1979). These results suggest that during this early critical period high levels of ecdysteroids are associated with worker development, and high levels of JH stimulate queen development.

11.3.3.2.2. The worker/soldier decision In contrast with the other highly eusocial Hymenoptera, division of labor within ant worker castes does not seem to be governed by age-related shifts in task performance. Rather, it is the relatively large size range of workers that underlies task preference and morphological specialization. Developmental mechanisms underlying worker caste polyphenism have been extensively reviewed (Wheeler, 1991) and mathematical models have been proposed that explicitly address the problem of growth rule reprogramming (Nijhout and Wheeler, 1994). Worker/soldier reprogramming is thought to take place during late larval instars in response to different feeding conditions or other forms of social influence on larval growth. These, in turn, appear to impact the larval endocrine system, which regulates growth and the onset of metamorphosis. Studies exploring this aspect were carried out on the red imported fire ant, *S. invicta*, which has a unimodal distribution of worker size (Wheeler, 1990), and on the strongly polyphenic *Pheidole bicarinata* (Wheeler, 1983, 1984).

Methoprene application to late larval instars led to an increase in size in *S. invicta* workers, probably due to a retarded onset of metamorphosis. In *P. bicarinata*, similar treatment resulted in the expression of soldier characters in the emerging brood, indicating a discrete JH-dependent developmental switch in the last larval instar. In species with a bi- or multimodal size distribution, larvae that reach a critical size, and consequently experience a different endocrine milieu, can thus be shunted into alternative developmental pathways leading to overt polyphenism, generally between a soldier and a worker caste (Wheeler, 1994).

While the role of JH seems to be fairly well established in *Pheidole* soldier/worker polyphenism, the primary triggering factors are still largely hypothetical. Two aspects could be relevant in this context. First is the larva/worker ratio, which determines the amount of nursing activity devoted to each larva and thus may affect body size (Porter and Tschinkel, 1985). The second factor is social pheromones, which inhibit the development of further soldiers once a soldier level appropriate to colony size has been reached (Wheeler, 1991). In terms of colony fitness, such regulatory mechanisms represent a generalized developmental basis for the concept of adaptive colony demographies postulated by Oster and Wilson (1978).

Similar to the queen/worker decision, a genetic basis has also been proposed for the observed polymorphism within the worker caste; for example, for the harvester ant *Pogonomyrmex badius* (Rheindt *et al.*, 2005; Smith *et al.*, 2008), the leafcutter ant *Acromyrmex echinator* (Hughes and Boomsma, 2007), and the army ant *Eciton burchelli* (Jaffe *et al.*, 2007). But there is a caveat in the proposals for genetic worker caste determination because these are all polyandric species, making it possible that the observed worker caste polyphenism may actually be the result of temporal variation in sperm genotypes that fertilize the eggs produced by the queen (Wiernasz and Cole, 2010).

Although our knowledge of the regulatory cascade from genetic, feeding, and/or pheromonal factors to endocrine activity and consequent programming of growth parameters is still fragmentary, a landmark study on gene networks underlying wing development in ants (Abouheif and Wray, 2002) has provided remarkable insight into downstream consequences of developmental reprogramming. In the wing buds of three ant species exhibiting different degrees of polyphenism, these authors investigated the expression patterns of six regulatory genes (*ultrabithorax, extradenticle, engrailed, wingless, scalloped*, and *spalt)*, all of which are functionally conserved in wing development of holometabolous insects. Cessation of wing bud development in worker-determined larvae is a characteristic element in all ants, and in species of the genus *Pheidole*

this cessation can occur in two steps. *Pheidole morrisi* soldier larvae develop large vestigial forewing discs but no visible hindwing discs, while worker-destined larvae develop neither of these wing discs. Of these six genes, all except for the most downstream one (*spalt)*, showed correlated expression patterns for the large forewing disc in soldier-biased larvae. None of these genes, however, were expressed in the forewing disc of worker larvae or in the hindwing discs of both soldier and worker larvae. Tracing the gene expression patterns through earlier development suggested (1) a gradual shutdown of the gene regulatory network occurring between mid-embryogenesis and the last larval instar and (2) that this patterning network was not interrupted at a single step, as might be expected under the concept of a classical switch mechanism. Instead, this study suggested that many different points in the gene expression cascade were involved. Extending the study to other ant species with lesser degrees of polyphenism (*Neoformica nitidiventris* and *Crematogaster lineolata*) corroborated the findings from *P. morrisi*. This led (Abouheif and Wray, 2002) to the conclusion that natural selection may be playing an active role in determining the most efficient route to halting wing development, and that, within the network, it may operate directly (independently) on different genes in different species.

This hypothesis has been followed up in the red imported fire ant, *S. invicta*, where vestigial wings were also discovered in worker larvae. These discs express the patterning genes *extradenticle, ultrabithorax*, and *engrailed* in accordance with expectations for normal wing development, but the wing discs do not grow (Bowsher *et al.*, 2007), inferring that wing disc patterning and growth are evolutionarily dissociated. This is a view that opens up exciting perspectives for hormonal effects on the expression of individual genes in the regulatory networks, and should stimulate the search for putative hormone response elements in the upstream control region of these genes in cross-species comparisons.

11.3.3.2.3. Queen polyphenism, queen loss, and queenless ants and wing dimorphism in males Notwithstanding its fascination, phenotypic diversity in the worker caste, which includes both continuous size variation and polyphenism, is restricted to only 15% of the ant genera and is mainly found in the highly derived groups. Yet, ants in the subfamilies Myrmeciinae and Ponerinae, which are characterized by a number of ancestral traits and are commonly believed to be socially less complex than the "higher" ants, are by no means lacking phenotypic variation. These subfamilies are of interest because they exhibit graded transitional series between queens and workers (Heinze, 1998), and workers in these ants are often not sterile, but may have large ovaries and possess developed spermathecae (Crossland *et al.*, 1988; Dietemann *et al.*, 2002; Ohkawara *et al.*, 1993). In some species, workers attain a queen-like morphology but contribute relatively little to colony reproduction, whereas in others, workers contribute substantially (e.g., the colony founding queen may be substituted by mated intercastes; Heinze and Buschinger, 1987; Peeters and Hölldobler, 1995).

In the most extreme cases, the queen caste has been completely lost, and all reproduction is carried out by workers, which are denominated gamergates (Peeters, 1991). Because in such queenless ants all females can potentially mate and lay eggs, a reproductive conflict of interest among colony females (as is typical in primitively social insects) is a secondarily evolved consequence. These species resolve the reproductive conflict by employing a mixed strategy of aggression by a dominant female and chemical signaling (Cuvillier-Hot *et al.*, 2001; Liebig *et al.*, 2000; Monnin and Ratnieks, 2001; Peeters *et al.*, 1999).

JH plays an interesting role in social dominance and fertility in these queenless ants. In contrast to primitively social wasps, where the JH titer positively correlates with dominance, and especially so with fertility (see Section 11.3.3.1.), application of the JH analog pyriproxyfen in queenless ants resulted in a *decrease* in fertility in the alpha female and in a loss in dominance rank (Sommer *et al.*, 1993). This decline in social rank was accompanied by corresponding changes in the cuticular hydrocarbon profile (Cuvillier-Hot *et al.*, 2004). Consequently, the idea that queenless ants represent a reversion to a primitive social system appears to be a superficial oversimplification that does not withstand a critical analysis once underlying developmental mechanisms and hormonal regulation of reproduction are taken into account. An interesting aspect in this context are the wing-disc derived gemmae of queenless ants, which deserve to be studied in the context of gene regulatory networks underlying wing development in ants.

Shutdown in wing development may not be exclusive to the female sex in ants. Recently this has also been shown to occur in males of *Cardiocondyla obscurior*, where a winged and a wingless male morph was found (Cremer *et al.*, 2002). Just as in workers, the loss of wings is not an isolated character state, but is part of a correlated modification in other characters, including reduced eye size. These "worker-like" males have a much prolonged period of spermatogenesis compared with winged conspecifics and mate exclusively inside the nest. This finding is a complete novelty and should stimulate comparative analyses on physiological and genomic events underlying concurrent polyphenism in the two sexes. Interestingly, both sexes responded with wing development when treated with a JH analog in a critical larval stage (Schrempf and Heinze, 2006).

11.3.3.3. Social bees Social systems with incipient caste differences between dominant egg-laying and subordinate females have originated multiply and

apparently independently within the Apidea (Michener, 2000). This happened in the Halictinae, the Xylocopinae, and of course most notably, the Apinae, which contain the closely related social tribes Euglossini, Bombini, Apini, and Meliponini.

11.3.3.3.1. The primitively eusocial bumblebees

Sociality in the bumble bees is obligatory and colonies can become quite large. The Bombini are large bees adapted to nesting in colder climates. Virgin queens emerge in autumn, mate, and hibernate before founding a new nest in spring. These queens are long-lived and survive for the entire seasonal nest cycle. The first brood emerging in early spring consists of a few rather small females (workers) that aid their mother in rearing the subsequent batches of exclusively female brood. Workers can vary considerably in size, and there is even slight overlap with queens in size-frequency distributions. During midsummer, the queen starts to lay haploid (male-producing) eggs, in addition to further female eggs. Soon after this "switch point," a few workers of high social rank also begin to lay (haploid) eggs, contributing to male production. The onset of worker reproduction has been termed the "competition point" (Duchateau and Velthuis, 1988), and marks an important incision in the colony life cycle, since from this point onward social integration deteriorates. By the end of the season, the colony produces large queens, which disperse and subsequently mate and hibernate. These components of the annual nest cycle have best been studied in *Bombus terrestris* (Röseler, 1985), and efforts in breeding and colony-rearing programs have been made to successfully introduce this species as a pollinator in greenhouses.

Although females produced before the competition point can attain a relatively large size, they never become queens (Cnaani *et al.*, 1997; Cnaani and Hefetz, 2001). This inhibition of queen production during the early stages of the colony cycle can theoretically be attributed either to "nutritional castration," that is, feeding of less or lower quality food to the respective larvae, or to a direct inhibitory effect from the egg-laying queen (Röseler, 1970). Long-term video recording of feeding acts, larval food analysis, and manipulation of feeding frequency (Pereboom, 2000; Pereboom *et al.*, 2003; Ribeiro, 1999; Ribeiro *et al.*, 1999) have now clearly established that nurse bees do not manipulate the larval feeding program; rather, nurses readily respond to hunger signals emitted by the larvae. Thus, it is the intrinsic feeding program of the larvae that is defined during the early larval instars.

The decision as to which feeding program the larvae will adopt has been attributed to a queen signal that inhibits larvae from developing into queens before the competition point in the colony cycle (Röseler, 1974). Although it is supposed to be a primer pheromone (Röseler *et al.*, 1981), its source and nature have not yet been unambiguously determined (Bloch and Hefetz, 1999b). Meticulous studies on the endocrine response elicited in the larvae pointed to the first and early second larval instar as the critical window for this queen signal (**Figure 11**). From the fifth day of the second instar, pre-competition-point larvae showed markedly reduced levels of JH synthesis (Cnaani *et al.*, 1997) and lower JH titers (Cnaani *et al.*, 2000b) when compared to queens. This response in the CA correlates with caste-specific differences in the ecdysteroid titer (Hartfelder *et al.*, 2000), indicating a synergistic interaction of the CA and prothoracic gland during the second and third larval instars. Interestingly, these hormone titer differences vanish during the feeding phase of the last larval instar when both castes exhibit low JH and ecdysteroid titers (Cnaani *et al.*, 2000b; Hartfelder *et al.*, 2000). Hormonal caste differences make their reappearance during the spinning and pre-pupal stages (Strambi *et al.*, 1984). In these early metamorphosis stages there is no quantitative modulation, only a temporal one in the position of the pre-pupal JH and ecdysteroid titer peaks (**Figure 11**).

Social conditions, especially the phase in the colony cycle, have a strong impact on the endocrine system, and thus on the switch from worker to queen development in the individual larvae. This was demonstrated when comparing JH release rates in larvae that were reared shortly before and shortly after the competition point (Cnaani *et al.*, 2000a).

This cascade from pheromonal effects on the feeding program to associated endocrine events in queen/worker differentiation does not necessarily represent a generalized chain of events in bumblebee caste polyphenism. Of the few comparative studies carried out on other bumblebee species, the most noteworthy ones were done on *B. hypnorum,* which was chosen for its different strategy in provisioning the larvae (Röseler, 1970). In this species, the queen/worker polyphenism involves a much later critical period. Larval fate is not determined during the early instars (as it is in *B. terrestris*); it is the feeding conditions in the last larval instar that appear to be the decisive factor. Yet again, the feeding differences converge in a caste-specific differential response in the endocrine system, and, as in *B. terrestris*, the pre-pupal JH and ecdysteroid titer peaks of *B. hypnorum* workers precede the corresponding peaks in queens (Strambi *et al.*, 1984).

As already mentioned, adult bumblebee workers and queens differ in size — despite some overlap in the bimodal size frequency distribution — and to some extent in their physiology, mainly in their energy metabolism (Röseler and Röseler, 1986), yet not in their capacity to lay eggs. Even in the presence of the queen, a considerable percentage of *B. terrestris* workers can exhibit signs of ovary activation. However, less than 40% of the workers complete oogenesis and these individuals do not lay eggs before the switch point in the colony cycle (Duchateau and Velthuis, 1989). Until this time point, egg laying in

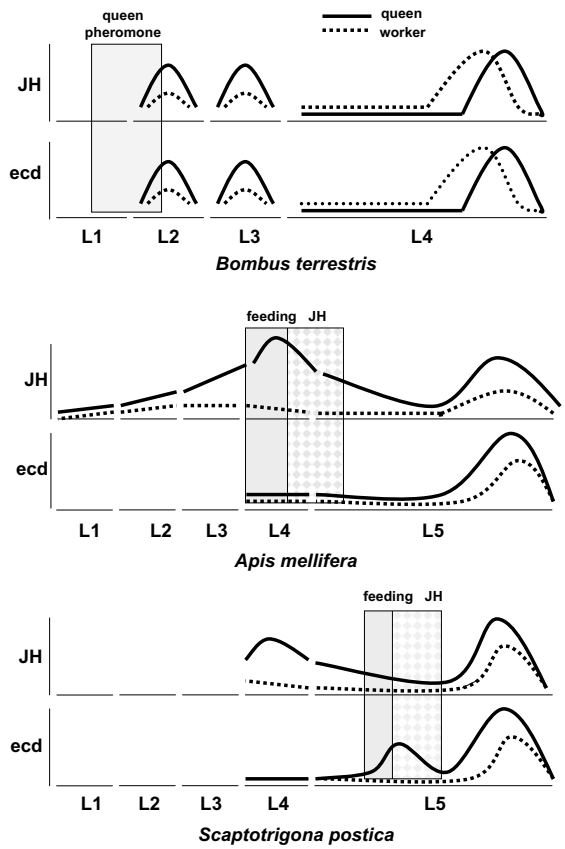

Figure 11 Juvenile hormone and ecdysteroid titer profiles in critical stages of caste development of the primitively eusocial bumblebee *Bombus terrestris* and the highly eusocial bees *Apis mellifera* and *Scaptotrigona postica*. In the primitively eusocial bumblebee, prospective queen and worker larvae differ in their JH and ecdysteroid titers during the early larval stages. Exposure during a critical period (shaded bar) to a repressor pheromone emitted by the egg-laying queen affects the hormone titers in the subsequent larval instars (L2 and L3). In the last larval instar (L4), the differences in hormone titers between queens and workers vanish and only a temporal shift in titer peaks is observed in the pre-pupal stage. In contrast, in the highly eusocial bees, *A. mellifera* and *S. postica*, the JH and ecdysteroid titers exhibit marked differences during the late larval stages. In *A. mellifera*, the major JH titer differences overlap with the nutritional switch from royal jelly to worker jelly in the fourth and early fifth instar (shaded bar). In *S. postica*, queen development is dependent on a prolonged feeding period (shaded bar) in the fifth instar. In *Apis* and in *Scaptotrigona* these critical stages overlap with a JH-sensitive period (dotted bar) established by JH application experiments. Morphological differences between queens and workers make their appearance in the highly eusocial bees but not in bumblebees. In the latter, queens differ from workers mainly in size and some physiological parameters. The expression of morphological caste differences thus appears to depend on sustained hormone titer differences in the last larval instar. Hormone titer profiles for *B. terrestris* were compiled from data by *Strambi et al.* (1984), *Cnaani et al.* (2000b), and *Hartfelder et al.* (2000). Data on *A. mellifera* were published by *Rembold* (1987) and *Rembold et al.* (1992), as well as by *Rachinsky et al.* (1990). Hormone titer curves for *S. postica* were adapted from *Hartfelder and Rembold* (1991). For data on critical periods and JH application experiments, see text.

workers is presumably inhibited by a queen-produced pheromone. The first studies pointing out a correlation between a queen inhibitory signal, JH production, and egg laying by workers were carried out by Röseler (1977) and Röseler and Röseler (1978). Reproductive workers had an elevated CA activity and JH titer relative to "non-activated" workers, and the queen inhibition of worker egg laying could be overcome by topical application of JH. Egg-laying inhibition by means of volatile pheromone was tested in a double-mesh screen assay and there was no evidence for such a signal (Alaux *et al.*, 2004), making it more likely that the workers autoregulate the onset of egg laying or eavesdrop on a queen signal that indicates that new queens will soon be produced in the colony (Alaux *et al.*, 2006).

A closer look at egg laying by workers revealed the existence of a dominance hierarchy within the worker caste in both queenright and queenless colonies. Dominant workers exhibit antagonistic behavior toward lower ranking workers, resulting in decreased CA activity and inhibition of egg laying in the latter ones (van Doorn, 1987). In small groups of queenless workers, a dominance hierarchy is quickly established, which is reflected in elevated JH titers in the high-ranking, egg-laying workers. The sequence in the response to queen removal was documented by monitoring JH synthesis, JH titer, ecdysteroid titer, and egg development during the first six days after queen removal and compared to queenright workers (Bloch *et al.*, 1996; Bloch *et al.*, 2000a,b). Levels of JH synthesis and JH titer were significantly elevated in workers three days after removal of the queen, followed by a similar increase in ecdysteroid titer, and larger terminal oocyte length at day six. This enhancement in JH release rates occurred independently of the colony cycle, that is, JH release was equally elevated in workers made queenless before and after the competition point (Bloch *et al.*, 1996). Yet, there was a strong correlation with worker age, since it was mainly the older workers that had elevated levels of JH release, and thus became dominant egg layers, both in queenless groups and in post-commitment point colonies. The absence of the queen is not the *sine qua non* for worker reproduction, since individually kept workers did not start egg laying and, correspondingly, maintained a low JH titer (Larrere and Couillaud, 1993). In accordance with previous studies on worker dominance hierarchies summarized by Röseler and van Honk (1990), it appears to be the high-ranking (older) workers that inhibit JH synthesis and ovary activation in lower ranking (younger) workers (Bloch and Hefetz, 1999a). Worker reproduction should be inhibited differentially during the two main phases of colony development, that is, prior to the competition point the queen inhibitory signal suppresses egg development in high-ranking workers, which acts synergistically with an inhibitory effect of dominant workers on ovary activation in the lower ranking workers. After

the competition point, it is primarily the high-ranking workers that control egg production in their nestmates. Both inhibitory signals appear to be mediated through the neuroendocrine axis, particularly via JH release. The elevated ecdysteroid titer observed concomitantly with progressive oogenesis (Bloch *et al.*, 2000b) should plausibly be interpreted as a consequence of the JH-stimulated ovary activation, yet one cannot exclude an ecdysteroid-mediated synergistic feedback effect on dominance as well (just as seen in *Polistes* wasps, see Section 11.3.3.1.).

Surprisingly, the strong correlation observed between JH titer and ovary activation in workers (Bloch *et al.*, 2000a) was not as striking in queens, which have lower JH titers than egg-laying, queenless workers (Bloch *et al.*, 2000a; Larrere *et al.*, 1993). A high JH titer is therefore not an absolute requirement for egg production, at least in queens, and may be more related to a combination of dominance status and egg production in the workers than with queen reproduction. Ecdysteroids may play a more important role in queen reproduction than in worker reproduction, as seen by the elevated ecdysteroid titers in overwintered queens, especially in the ovaries (Bloch *et al.*, 2000b; Geva *et al.*, 2005). Thus it seems that in bumblebees we are confronted with a split in the role of JH and ecdysteroids in female reproduction, with JH playing the prominent role in workers and ecdysteroids in queens. An interesting question to ask is whether or not and how diapause physiology (see Chapter 10) may play a role in setting up this split in the endocrine system of reproductive physiology in the bumblebee castes, both ontogenetically and in evolutionary terms. Clear diapause effects on development and physiology are seen in butterfly phase (Section 11.3.1.2.1.) and other caste polyphenisms (Sections 11.3.3.1. and 11.3.3.2.).

11.3.3.3.2. The highly eusocial honey bees and stingless bees Honey bees (Apini) and the closely related Meliponini, colloquially referred to as stingless bees, have been companions of mankind for almost as long as locusts. Their origin and social organization has been narrated in picturesque myths, and their biology is a well-represented element in ethnobiology. The common themes to these myths are the nature of social integration and the origin of the differences in form and function between queens and workers.

One of the main difficulties in explanations for queen/worker dimorphism, both for myths and the modern scientific approach, resides in the identification of the mode of action for the initial triggers in caste development. As in most social insects, this is a problem of larval nutrition. In the honey bees, larvae are continuously fed during larval development, and during the early larval instars, both queen and worker larvae receive mainly a glandular proteinaceous secretion (royal jelly). In the fourth and fifth larval instar this type of food is provided in large quantities only to queen larvae, whereas worker-destined larvae are fed a mixture of glandular secretions, honey, and pollen. Apart from the switch in diet type, worker larvae are also visited and fed much less frequently than queen larvae (Beetsma, 1985). Attempts to identify specific queen-determining factors in royal jelly resulted in a partial purification of chemically labile factors (Rembold *et al.*, 1974b), but unequivocal evidence for a "queen determinator" was not obtained, leading these authors to consider the requirement for a balanced diet. (But see Note added in proof).

This view is also consistent with results of larval food analyses in stingless bees (Hartfelder and Engels, 1989), which do not progressively feed their brood; instead, they mass provision the brood cells shortly after they are built. Due to mass provisioning of the brood cells and lack of further interaction of worker bees with the developing brood, other than regulation of the colony microclimate, there is no evidence for a nutritional switch in the stingless bees that could serve as a signal for queen/worker development. In the majority of the stingless bee species (i.e., those pertaining to the highly diverse trigonine genera), queen development depends on the quantity of larval food provided to the larvae, and this is reflected in the size of the cells. Large queen cells containing two- to threefold more larval food than the worker cells are often built at the margins of the horizontal brood combs (Engels and Imperatriz-Fonseca, 1990) or result from the fusion of two brood cells sitting on top of one another, thus providing a larva with a second portion of larval food. This strategy of queen production is also observed in the context of emergency queen rearing in some species when a colony has lost its queen (Faustino *et al.*, 2002).

An exception to the queen/worker determining mechanism by modulation of larval food quantity is the genus *Melipona*. Their brood cells are of equal size, irrespective of whether queens, workers, or males are reared within them. Up to 25% of the female brood can be queens, which led Kerr (1950) to propose the hypothesis of a genetic predisposition with a two-locus, two-allele system where only double heterozygotes can become queens. Even in this system, larval food quality has a modulating effect on caste differentiation (Kerr *et al.*, 1966), since maximal levels in queen production are only observed in strong colonies that have sufficiently large stocks of honey and pollen. Under suboptimal conditions, the proportion of queens in the female brood is generally well below 25%. An alternative hypothesis based on modeling optimal queen production frequencies in the queen/worker conflict attributes "self-determining" capacity to the larvae (Ratnieks, 2001). Although the two hypotheses are not mutually exclusive, because one addresses proximate mechanisms and the other ultimate causes, both are awaiting empirical validation. This may be obtained through genetic marker analyses, for example, by means of linkage in AFLP analyses (Hartfelder *et al.*, 2006; Makert *et al.*,

2006). Irrespective of the distinct modalities in initial triggers (i.e., nutritional switch in honey bees, large food quantities in trigonine bees, or a genetic predisposition in *Melipona* species), all of these inputs eventually converge in caste-specific activity patterns in the endocrine system, which govern subsequent differentiation events in the target tissues.

11.3.3.3.2.1. The endocrine regulation of caste development in honey bees. This endocrine cascade has been best explored in the honey bee. JH application experiments carried out in the 1970s indicated an eminent role for JH in caste development, and established a critical window for its action in the fourth and early fifth larval instar (Dietz *et al.*, 1979; Rembold *et al.*, 1974a; and further references in Hartfelder, 1990). Subsequent analyses of endogenous JH titers by specific radioimmunoassays and highly sensitive GC-MS corroborated these experimental JH effects, revealing a marked difference in JH titers between queen- and worker-biased larvae (**Figure 11**) during larval, pre-pupal and the late pupal stages (Rachinsky *et al.*, 1990; Rembold, 1987; Rembold *et al.*, 1992). From these meticulous JH titer analyses it was possible to ask how the JH titer differences may be generated and especially, how they may affect caste-specific differentiation processes in target tissues. Analyses of JH biosynthesis and release rates by means of a radiochemical assay revealed a markedly elevated CA activity in prospective queen larvae between the fourth and the early fifth larval instar (Rachinsky and Hartfelder, 1990), inferring that the JH titer differences are primarily a result of differential CA activity. Rates of JH degradation were generally low in honey bee development (Mane and Rembold, 1977) and are primarily controlled by a recently identified honey bee JH esterase (Mackert *et al.*, 2008). A JH epoxy hydrolase-like gene was also identified in the honey bee genome, but functional assays showed that it does not degrade JH (Mackert *et al.*, 2010).

An interesting facet was the finding that the terminal steps in JH biosynthesis are the critical ones for the differential JH release rates in early fifth instar queen and worker larvae (Rachinsky and Hartfelder, 1991; Rachinsky *et al.*, 2000). Blocking the terminal steps in JH synthesis, from farnesoate to JH III, is an efficient means to generate and guarantee low JH titers in worker larvae during the fourth and early fifth instar. Furthermore, since this block is reversible, as required to generate the elevated JH titers in pre-pupae (Rachinsky and Hartfelder, 1990, 1991), the ortho-methylfarnesoate transferase and the JH-epoxidase are candidate targets for allatoregulatory factors. Radiochemical assays on CA of worker larvae showed that *M. sexta* allatotropin (Manse-AT) can stimulate JH synthesis in honey bees in a dose-dependent manner, yet does not overcome the block on the terminal steps (Rachinsky and Feldlaufer, 2000; Rachinsky *et al.*, 2000). Immunolocalization for Manse-AT-like material in honey bee brains detected a discrete, small number of cells in pre-pupae, but not in the earlier stages of honey bee development, making it difficult to assert that a Manse-AT-like peptide is involved in the regulation of CA activity during the critical stages of caste determination. CA regulation becomes even more complex considering that a set of peptides from the brain and subesophageal ganglion (Rachinsky, 1996) as well as biogenic amines, especially serotonin (5HT) and octopamine (Rachinsky, 1994), may modulate JH biosynthesis to generate the observed caste-specific profile.

This search for allatoregulatory factors represents an upstream walk to close the gap between the initial nutritional signal that induces caste development and its translation into an endocrine response. The only pathways mapped in this context are the serotonergic (Boleli *et al.*, 1995; Seidel and Bicker, 1996) and the stomatogastric nervous system (Boleli *et al.*, 1998). Immunocytochemical mapping of serotonergic neurons in honey bee larvae and pupae revealed an apparent heterochrony in their development. Whereas serotonergic neurons in the ventral ganglia exhibited a seemingly mature architecture already in the larval stages, 5HT-neurons in the brain matured only during late pupal development (Boleli *et al.*, 1995). These results seemingly preclude an allatoregulatory function for protocerebral 5HT-neurons during pre-imaginal development. Instead, the detection of immunoreactive cell bodies in the vicinity of the CA, close to their connection to the *nervi corporis cardiaci III*, implicates the stomatogastric nervous system in the modulation of CA activity. Since the stomatogastric nervous system provides a direct link between sensory cells for food quality in the labral region of honey bee larvae (Goewie, 1978), and the food-intake-mediating neurons of the stomatogastric nervous system integrate with the retrocerebral endocrine complex (Boleli *et al.*, 1998), the nutritional switch information could certainly take this (alternative, or even more direct) pathway. Nevertheless, information on perception and processing of the nutritional switch signal in bees is still rudimentary and remains a major black box in our understanding of events upstream of the caste-specific endocrine responses.

One of the downstream effects resulting from differences in JH titers between honey bee queen and worker larvae involves the endocrine system functioning as an internal circuit between the CA and the prothoracic glands. The latter were identified in honey bee larvae as loose agglomerates of cells projecting from the foregut to the retrocerebral complex (Hartfelder, 1993), and their activity pattern closely reflects the ecdysteroid titer in the last larval instar (Rachinsky *et al.*, 1990). The ecdysteroid titer, with makisterone A as the main compound, is low at the beginning of the last instar (**Figure 11**), and exhibits a marked peak during the pre-pupal stage in both castes. Caste-related differences in the larval ecdysteroid titer are evident in the cocoon-spinning phase, with an earlier

increase in queens than in workers. Mimicking a queen-like JH titer by an exogenous JH application to worker larvae in the fourth to early fifth instar resulted in a precocious increase in prothoracic gland activity (Rachinsky and Engels, 1995). This positive interaction between JH titer and prothoracic gland activity may involve a prothoracicotropic hormone (PTTH)-like factor, which was detected immunohistochemically in honey bee brain sections (Simões *et al.*, 1997).

Alternatively, JH could directly stimulate ecdysteroid synthesis and release in the prothoracic glands, as demonstrated by *in vitro* assays exposing these glands to different doses of methoprene (Hartfelder and Engels, 1998). The positive action of JH on the ecdysteroid titer in worker larvae seems to be restricted to the larval–pupal transition, since JH application to pupae inhibited and retarded the formation of the large pupal ecdysteroid peak (Zufelato *et al.*, 2000), which also mainly consists of makisterone A (Feldlaufer *et al.*, 1985).

In terms of target tissues, it is the ovary that has received the most attention, because it best reflects the functional caste differences. A typical queen ovary consists of 150–200 ovarioles, whereas a worker ovary most often contains between 2 and 12 of these serial units. These differences arise primarily during the final larval instars, even though some of the differentiation steps are already visible as early as the second or third instar (Dedej *et al.*, 1998; Reginato and Cruz-Landim, 2001). Caste-specific differentiation of the ovary consists primarily of a reduction in ovariole number in the worker caste, from an initially equal number of ovariole anlagen in the two castes — generally over 150 anlagen per ovary in the fourth instar — to the final set of ovarioles at pupation. Histologically, this process is marked by a reduced number of rosette-like cystocyte clusters and a large number of autophagic vacuoles in early fifth instar worker ovarioles (Hartfelder and Steinbrück, 1997).

The reduced number of cystocyte clusters is not due to a diminished mitotic activity in the ovaries of worker larvae (Schmidt Capella and Hartfelder, 1998); rather it results from a disintegrating actin cytoskeleton in the germ cells (Schmidt Capella and Hartfelder, 2002). These degradative processes initiated by actin-spectrin dissociation could be reverted by a single topical JH application to late fourth instar workers (Schmidt Capella and Hartfelder, 1998; Schmidt Capella and Hartfelder, 2002). Even though these results permit us to pinpoint a cell biological target for JH in an important caste differentiation process, we cannot yet establish whether JH acts directly on the affinity of actin to spectrin, or whether this is due to a transcriptional effect on factors that mediate the interaction between these cytoskeletal components.

A completely different approach toward understanding the development and evolution of the tremendous difference in ovary size between queen and worker honey bees comes from quantitative trait loci (QTL) mapping (Linksvayer *et al.*, 2009). This study identified a series of interesting candidate genes, such as *quail*, a gene involved in actin filament-dependent apoptosis in nurse cells in the *Drosophila* ovary, *cabut* an ecdysteroid-responsive transcriptional activator also associated with cell death, *delta*, the main player in Notch signaling, and *miro*, which is involved in mitochondrial homeostasis, apoptotic signal transduction, and cytoskeleton organization. Taken together, these results nicely illustrate the convergence of results coming from hypothesis-driven and non-biased research approaches, both pointing to the importance of cytoskeletal organization and cell signaling in the caste-specific differentiation of the honey bee ovary.

11.3.3.3.2.2. Honey bee caste development — Insights from genomic studies. As in functional genomics in general, complex transcriptional regulation can be expected to be a key factor in our comprehension of caste development, and in this respect, the honey bee stands out as a model organism among the social insects. The early studies on mRNA differences between queen and worker larvae (Severson *et al.*, 1989) have gained considerable depth and impetus from powerful molecular methods generating differentially expressed EST (Corona *et al.*, 1999; Hepperle and Hartfelder, 2001) and suppression-subtractive hybridization libraries (Evans and Wheeler, 1999, 2000). By clustering algorithms three major transcriptomic groupings became evident, a dichotomy for early versus late larval stages and a queen-worker dichotomy in gene expression that begins in the late larval stages. A major breakthrough in honey bee genomic analysis finally came with the publication of the complete and annotated honey bee genome (The Honey Bee Genome Sequencing Consortium, 2006). Against this database, the caste specifically expressed ESTs of the earlier studies could now be interpreted in context. A Gene Ontology-based annotation (Cristino *et al.*, 2006) inferred that metabolic regulation must be a major factor in caste development, and one gene that appears to be an important player herein encodes an ecdysone-responsive short chain dehydrogenase/reductase (Guidugli *et al.*, 2004). Motif searches for putative transcription factors in upstream control regions of differentially expressed genes then set the road map to the reconstruction of genomic networks acting in honey bee caste development (Cristino *et al.*, 2006). Such networks became comprehensive once larger data sets of caste- and stage-specific gene expression became available by means of microarray analyses (Barchuk *et al.*, 2007). As shown in **Figure 12**, the genomic regulatory networks derived for queen and worker development show strongly divergent topographies, denoting to the complexity in gene expression shifts during post-embryonic caste differentiation. Furthermore, network nodes are of heuristic value as they pinpoint genes at crucial connections within the network, which should be the focus of further in-depth studies.

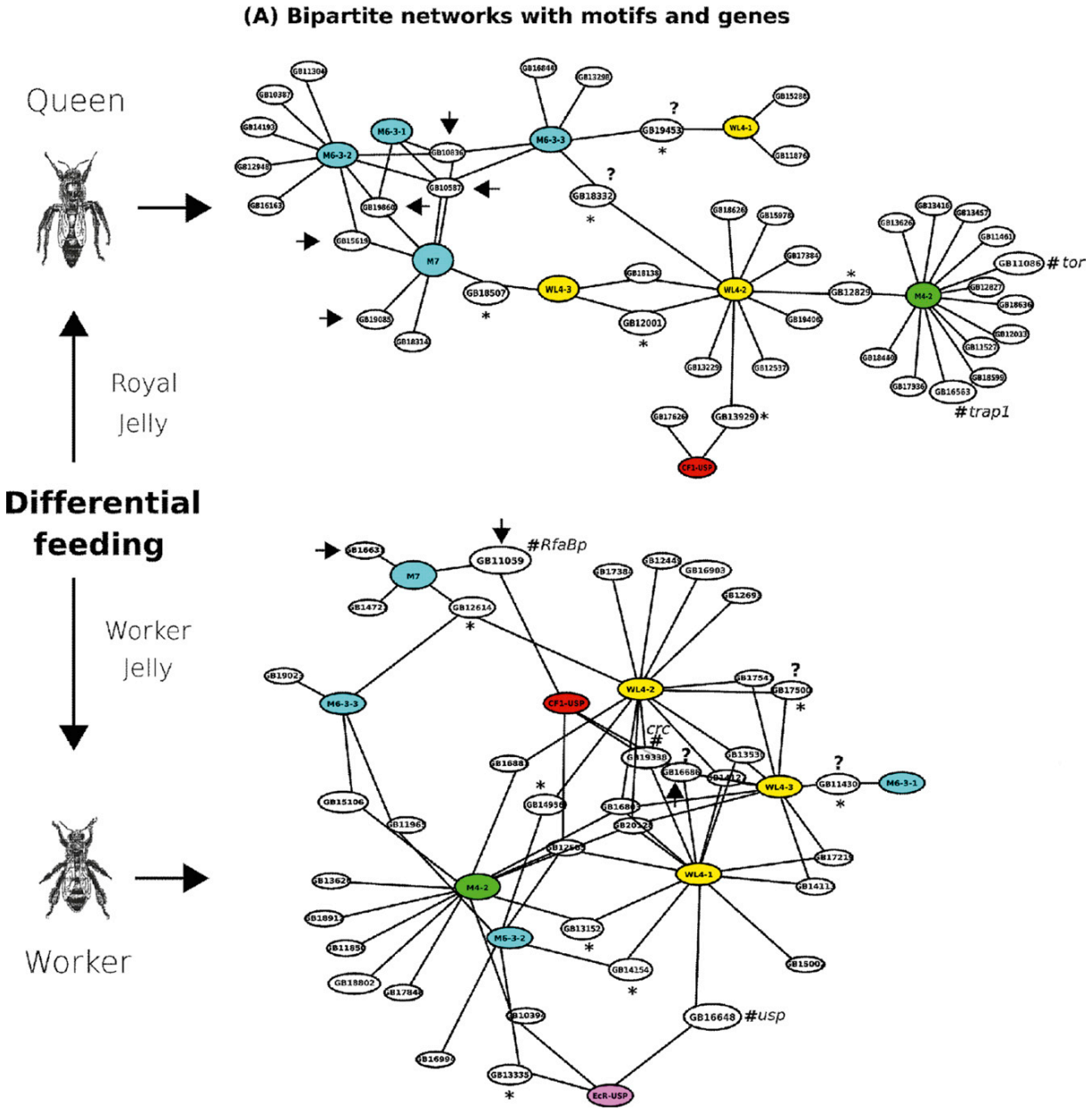

Figure 12 Network analysis depicting putative gene interaction in caste development of the honey bee, *Apis mellifera* (*Barchuk et al.*, 2007). Differential gene expression data were obtained by microarray analysis of third, fourth, and early fifth instar queen and worker larvae. After collecting Gene Ontology attributes for differentially expressed genes, their respective upstream control regions (UCRs) were retrieved from the genome sequence and were screened for putative regulatory motifs by means of a Gibbs sampling approach integrated into a motif discovery and analysis script (*Cristino et al.*, 2006). Functional ecdysone responsive elements and Ultraspiracle (USP) binding site information was obtained from studies on *Drosophila*. Motifs represented in blue were associated with JH responsive genes in caste development, those in green with cell death events. Yellow circles represent a set of ten genes that are the most overexpressed in workers compared to queens, and in magenta are motifs related to ecdysone response and USP. The black arrows point to genes consistently upregulated in caste development and in JH application assay. Genes with unknown function are marked by a question mark. Genes marked by an asterisk were not in the training data set for motif discovery. The worker differentially expressed genes marked by a hash (#) are *usp*, *crc*, and *RfaBp*, which are repressed by hormones. The queen differentially expressed genes marked by a hash (#) are *tor* and *trap1*, the latter being a negative regulator of apoptosis in response of nutrition. The more interconnected network obtained for worker larvae suggests that the differentially expressed genes share a larger number of *cis*-elements than the queen differentially expressed genes. Reproduced with permission from *Barchuk et al.* (2007).

Alternative approaches to the non-hypothesis-driven expression library or microarray analyses are candidate gene approaches. Because honey bee queens and workers greatly differ in size, factors controlling growth should be important. Consequently, several studies have now investigated the functionality of the insulin-insulin-like signaling (IIS) pathway together with its parallel branch, the target of rapamycin (TOR) pathway. The clearest results were obtained for *Apis mellifera* TOR gene function. *Amtor* expression is higher in young larvae destined to become queens, and a knockdown experiment on AmTOR function by feeding *Amtor* dsRNA to larvae reared *in vitro* inhibited the development of queen characters (Patel *et al.*, 2007). These results established a positive function for *Amtor* in queen development, in accordance with predictions form the *Drosophila* model. The role of the IIS pathway, on the other hand, turns out to be considerably more complex. This is because there are two insulin-like peptides (AmILP1 and AmILP2) and two insulin receptors (AmInR1 and AmInR2) predicted in the honey bee genome (Azevedo and Hartfelder, 2008; Watt, 1968; Wheeler *et al.*, 2006), and especially in the late larval stages, when the growth rates of queen larvae tremendously surpass those of workers, the expression of the AmILP2 encoding gene is, surprisingly, more expressed in worker than in queen larvae, and this ILP gene is the most expressed gene among the two predicted genes. Furthermore, the expression of both insulin receptor genes was downregulated in queen larvae while they exhibited enhanced growth (Azevedo and Hartfelder, 2008). Honey bees, thus, differ from the *Drosophila* model (Colombani *et al.*, 2005; Mirth *et al.*, 2005; Oldham and Hafen, 2003) when it comes to IIS-mediated growth control, thus representing an apparent paradox.

It is possible that the downregulation in IIS function may be contingent on the JH titer, which also shows a decrease during this phase in queen larvae, but searching for further interacting factors indicated that the hypoxia signaling pathway could be involved. A possible role for the involvement of oxidative metabolism in honey bee caste development had already been indicated from differential gene expression screens, but evidence dated back to very early studies on respiratory rates in honey bee larvae (Melampy and Willis, 1939) and investigations on mitochondrial functions, especially cytochrome c content (Eder *et al.*, 1983). Since the three hypoxia signaling core genes turned out to be highly conserved in the honey bee genome, it was possible to study their transcript levels during queen and worker development. It became clearly apparent that worker larvae strongly overexpress these genes (Azevedo *et al.*, 2011), even though ambient oxygen availability in the hive should be the same for queen and worker larvae. These results from non-biased microarray studies, as well as from candidate gene approaches, now pinpoint the importance of metabolic regulation to caste development.

A further intriguing insight into mechanisms underlying caste development comes from the observation that the honey bee genome, distinct from *Drosophila melanogaster*, has a full complement of DNA methylating enzymes (The Honey Bee Genome Sequencing Consortium, 2006). Using this genomic information, an RNAi-mediated knockdown of the DNA methyltransferase *Dnmt3* gene caused worker-destined larvae to express queen-like characters, such as large ovaries (Kucharski *et al.*, 2008). This was a surprising insight into the unexpected tremendous role that epigenetic regulation exerts on the honey bee transcriptome, and current studies are directed toward analyzing the genome-wide role of methylation in honey bee queens and workers (Foret *et al.*, 2009) and imprinting. Preliminary results indicate that large portions of the honey bee genome may be imprinted (G. Hunt, personal communication).

11.3.3.3.2.3. Honey bee reproduction and division of labor. The high rates of egg production in the honey bee queen require an enormous production of vitellogenin by the fat body. In contrast to most insects, hormonal regulation of vitellogenin expression in honey bees is rather puzzling. Juvenile hormone application experiments, allatectomy, and also JH titer analyses all test against a role for this hormone in the regulation of the vitellogenin titer in adult queens (Engels, 1974; other references cited in Hartfelder and Engels, 1998). Rather, the gonadotropic function of JH, especially the stimulation of vitellogenin expression, seems to have been shifted to the late pupal stage (Barchuk *et al.*, 2002), and strikingly, vitellogenin transcripts were already detected during larval development (Guidugli *et al.*, 2005b). As in other insects, much of the mysteries of JH action can be ascribed to a lack of insights into its receptor and downstream mode of action. In the honey bee, a homologue to Ultraspiracle, which is considered an insect JH receptor candidate, has been identified as a single copy gene that is upregulated in response to JH (Barchuk *et al.*, 2004). Furthermore, knockdown of this gene by RNAi delayed pupal development, but since it did not affect vitellogenin gene expression during this phase (Barchuk *et al.*, 2008), its function in the JH response cascade is far form clear. As indicated from functional studies on a JH response element (DmJRE1) that was found in the promoter region of some of the JH-induced honey bee genes, proteins interacting with this element were shown to also interact with the ecdysone receptor and Ultraspiracle gene products (Li *et al.*, 2007), thus inferring a cross-talk between JH and ecdysteroid signaling. This interaction could be especially relevant for vitellogenin induction in the late pupal phase.

In contrast to JH, no apparent role in reproduction and division of labor in female honey bees can be attributed to ecdysteroids. Except for a transient small peak early in the adult life cycle, ecdysteroid titers fluctuate at basal levels, independent of caste, reproductive status, and colony social conditions (Hartfelder *et al.*, 2002). This is all the

more striking when considering that considerable levels of ecdysteroids were detected in the ovaries of adult queens (Feldlaufer *et al.*, 1986). Furthermore, a homologue of the ecdysteroid-regulated gene *E74* was shown to be selectively expressed in the ovaries of queens and in mushroom body interneurons of adult workers (Paul *et al.*, 2005).

Caste and reproduction in the highly eusocial insects are intimately related to life span, and contrary to standard animal models, high fertility is associated with extended life span (Heinze and Schrempf, 2008) or more accurately, with delayed aging, also making the honey bee a model system of particular interest to the medical field (Münch *et al.*, 2008). Notwithstanding the complexity of the aging and senescence syndrome, an apparently simple regulatory circuitry based on a mutual negative feedback of JH on the vitellogenin titer and vice versa emerged from initial modeling studies (Amdam and Omholt, 2002, 2003). These built on early evidence showing that the application of JH to young worker bees promotes a precocious switch from activities within the nest, especially brood care, to foraging activities typical of older workers (Jaycox, 1976). Subsequent analyses of JH titers and CA activity confirmed these data, showing that rates of JH synthesis and hemolymph JH titers in worker bees strongly increase as these individuals age and become foragers (Huang *et al.*, 1991; Robinson *et al.*, 1991, 1992). This switch from within-hive to foraging (out-of-hive) activity has long been known to be accompanied by a decrease in hemolymph vitellogenin levels (Engels, 1974), but the functional relevance of this mutual negative feedback only became apparent once novel properties of vitellogenin emerged, in addition to its primary role as a yolk protein precursor. Honey bee vitellogenin turned out to also be a major zinc transporting protein and to directly affect immunosenescence of hemocytes (Amdam *et al.*, 2005). Such age-related immunosenescence was further corroborated through an analysis of various stressor effects, showing that older workers were less resistant to stress than younger bees (Remolina *et al.*, 2007).

For proof of principle of the postulated mutual negative feedback between JH and vitellogenin (double repressor hypothesis), it was essential to show that not only does JH represses vitellogenin synthesis (Engels *et al.*, 1990), but also that downregulating vitellogenin expression may, in turn lead to an increase in the hemolymph JH titer. This has now been shown in several experiments using RNAi-mediated knockdown of vitellogenin gene function (Amdam *et al.*, 2005; Guidugli *et al.*, 2005a; Marco Antonio *et al.*, 2008; Nelson *et al.*, 2007). Since the interplay between JH and vitellogenin affected a whole suite of behavioral characters, including gustatory responses and preferences between pollen and nectar (Amdam *et al.*, 2006b; Tsuruda *et al.*, 2008), this circuitry is considered to represent a key module in the division of labor in a honey bee colony. Viewed against the background of social insects having evolved from non-social ancestors, this circuitry has been formulated as the "reproductive ground plan hypothesis" (Amdam *et al.*, 2004).

A major open question herein is the molecular structure of the vitellogenin signaling pathway. A vitellogenin receptor candidate gene was shown to be expressed in honey bee fat body, ovaries, and also in the brain (Guidugli-Lazzarini *et al.*, 2008), consistent with findings for vitellogenin expression (Corona *et al.*, 2007). In addition, a signal originating from the ovaries was recently postulated based on ovary transplantation experiments (Wang *et al.*, 2010). Furthermore, a cross-talk of vitellogenin signaling with the IIS pathway has been observed (Corona *et al.*, 2007). In addition, high-throughput and quantitative RT-PCR gene expression analyses of worker honey bee brains further singled out the importance of nutrition-mediated signals acting through the IIS pathway (Ament *et al.*, 2008, 2010), and emphasized the role of the brood pheromone in orchestrating large-scale transcriptional responses in worker bees (Alaux *et al.*, 2009).

With all this evidence adding to the heuristic power of the apparently simple reproductive ground plan hypothesis, the complexity of its genetic architecture emerged from the genomic annotation of the pln QTL (Hunt *et al.*, 2007a) that contribute to pollen hoarding behavior and could be analyzed through a long-term selection program for this trait. In these QTL studies, genes related to ovarian development and the IIS pathway were detected as over-represented. Furthermore, evolutionary transitions in social insects, as related to the reproductive ground plan, do not seem to be restricted to the female sex, because vitellogenin and its receptor are also expressed in different tissues of honey bee drones (Colonello-Frattini and Hartfelder, 2009; Trenczek *et al.*, 1989).

To conclude, the reproductive ground plan hypothesis (Amdam *et al.*, 2004), which builds upon the prior ovarian ground plan hypothesis (West-Eberhard, 1996), hinges on the remodeling of (1) an ancestral JH-vitellogenin circuitry regulating oogenesis and (2) the segregation of behaviors — that ancestrally are associated with a reproductive phase (foraging and brood rearing with a protein/lipid-rich diet) and a non-reproductive phase (foraging for carbohydrate-rich food) in the life history of solitary wasps or bees — into two functionally and morphologically distinct castes, such as queens and workers. The hypothesis is well supported for temperate-climate paper wasps (Hunt *et al.*, 2007b) and bees, especially the honey-bee (Amdam *et al.*, 2006a; Amdam and Page, 2007) and has come to be a powerful framework in which to explain caste polyphenism in social Hymenoptera because these polyphenisms are all centered around reproduction. A possible extension to termites has not yet been explored.

11.3.3.3.2.4. Hormonal control of caste development and reproduction in stingless bees. The stingless bees are not only closely related to the monogeneric tribe Apini, but

are also represented by an enormous number of species (Camargo and Pedro, 1992). Thus, they supply us with ample material for evolutionary insights into caste polyphenism and reproduction in highly social bees. As previously mentioned, it is the nutritional conditions that serve as the initial trigger in the divergence of the queen/worker developmental pathways, even though there is strong evidence for a genetic predisposition to caste fate in the genus *Melipona* (see Section 11.3.3.3.2.).

As in the honey bee, the initial investigations on the role of hormones in stingless bee caste differentiation all relied on JH application experiments. These comparative investigations on different species of stingless bees (Bonetti *et al.*, 1995; Buschini and Campos, 1994; Campos, 1978, 1979; Campos *et al.*, 1975) established the spinning stage of the last larval instar as the critical phase for JH-dependent induction of queen development. These findings subsequently received support from investigations on CA activity and JH titer measurements in *Scaptotrigona postica* (Hartfelder, 1987; Hartfelder and Rembold, 1991). These results indicated that JH-dependent differentiation steps in queen development occur much later in stingless bees when compared to the honey bee (Hartfelder, 1990; **Figure 11**). Also, queen development in the trigonine species takes longer than worker development, with the opposite rue for honey bees and the genus *Melipona*, indicating that the differences in nutritional programs among the three groups (*Apis*, *Melipona*, *Trigonini*) are reflected in larval and especially pupal ecdysteroid titers (Hartfelder and Rembold, 1991; Pinto *et al.*, 2002), arguing for a correlated regulation of caste development by JH and ecdysteroid in these groups of highly social bees.

The high species diversity in the stingless bees, their variation in colony size, nesting sites, and a large number of further aspects in social lifestyles, obviously provide ample material for variation on the basic themes of caste development, reproduction and division of labor, and possible hormonal regulation therein. An important difference between honey bees and stingless bees lies in the degree of morphological differences between the sexes and castes. The males of stingless bees are morphologically much more similar to workers than they are to queens (Kerr, 1987, 1990), even though growth rules for the different morphogenetic fields along the body axes appear to be adjusted rather independently (Hartfelder and Engels, 1992).

A striking phenomenon calling for functional explanations is the strong variation in queen size in some species. The occurrence and reproductive performance of miniature queens was closely studied and compared to normal-sized queens (Ribeiro *et al.*, 2006), indicating functional differences in reproductive performance related to colony conditions.

Since monopolization of reproduction by the queen is a key element in the social evolution of bees, the large variation in reproductive activities by the workers among stingless bee species can provide insight into different evolutionary solutions to the queen–worker and also the worker–worker conflict over reproduction. Worker oviposition can take two forms: (1) trophic eggs that are laid shortly before the queen oviposits and (2) reproductive eggs that are laid after the queen's oviposition. Trophic eggs are unviable eggs that are specially produced as nutrition for the queen serving to maintain her high reproductive rates. Worker vitellogenin is, thus, directly shunted into egg production by the queen. Since the production of (trophic) worker eggs is clearly in the interest of the queen, she does not discourage workers to produce these unviable eggs and thus should keep the workers' ovaries in an active state. It is therefore not surprising that workers can also produce viable eggs that can make a significant contribution to male production in a colony (Cepeda, 2006; Engels and Imperatriz-Fonseca, 1990; Velthuis *et al.*, 2005). Some species, such as *Frieseomelitta varia* have, however, opted for a completely different solution to this conflict over reproduction, as their workers are completely sterile due to the complete degeneration of their ovariole anlagen during pupal development (Boleli *et al.*, 1999, 2000). All the more surprising, on first sight, but consistent with the evolutionary transitions implicit in the reproductive ground plan hypothesis, vitellogenin expression in this ovary-less stingless bee was practically constitutive both at the transcript and at the protein level (Dallacqua *et al.*, 2007; Hartfelder *et al.*, 2006).

The involvement of hormones in the regulation of reproduction and division of labor in adult stingless bee queens and workers has only marginally been investigated, and so far there is only negative evidence to this end in *Melipona quadrifasciata*. As in the honey bee, ecdysteroids do not seem to play any role in queen or worker reproduction in *M. quadrifasciata* (Hartfelder *et al.*, 2002). Interestingly, however, analyses of *ultraspiracle* gene homologues in *M. scutellaris* and *S. depilis* (Teles *et al.*, 2007) showed differences in pupal transcript levels that might be related to differences in reproductive strategies seen in adult workers of trigonine and *Melipona* species (Velthuis *et al.*, 2005).

Genomic resources are still scarce for stingless bees, with the exception of *M. quadrifasciata*, for which a suppression subtractive library analysis has revealed 337 unique sequences as differentially expressed between newly emerged queens and workers (Judice *et al.*, 2006). This situation is, however, bound to change as efforts are under way to generate 454 sequence data for at least two stingless bee species.

11.4. Synthesis and Perspectives

In the previous sections, we presented insect polyphenisms and detailed the underlying ontogenetic mechanism in a phylogenetic context to facilitate drawing parallels to general modes of development and reproduction in hemi- and holometabolous insects. The most common forms of

polyphenism are built around the framework of dispersal and reproduction. Considering that the development of wings has played a major role in the extraordinary evolutionary and ecological radiation of insects, it appears that aspects of wing formation have proven especially amenable to the evolution of facultative or polyphenic patterns of wing expression. The switch between winged and wingless developmental pathways most often occurs during a late nymphal instar and frequently involves modulation of the JH titer during a critical physiological "sensitive period." Research on wing polyphenism in cricket species has not only firmly established a key role for JH in wing size variation, but also called attention to the fact that it is JH degradation by a JHE and not CA activity that is the controlling factor of the JH titer modulation in nymphs. In contrast, in adult crickets, wing length is associated with an interesting diurnal variation in JH synthesis in the long-winged morph, and genetic variation underlying this trait is a prime example of the interaction of genotype and environment (population density) in the expression of an adaptive life history polyphenism (Roff and Fairbairn, 2007; Zera *et al.*, 2007).

When compared to crickets, phase polyphenism in locusts is a much more complex syndrome involving transgenerational transfer of information in the transition from the solitary to the gregarious morph. Whereas a role for the endocrine system is best established for changes in body pigmentation, with high levels of JH promoting expression of the green background color of the solitary morph and the neuropeptide [His^7]-corazonin the dark foreground pattern, the physiological factors underlying the shift in other aspects of the syndrome (leg and wing morphometry, ovariole number, and behavior) are less well understood (Pener and Simpson, 2009). A much debated issue for understanding the phase shift in migratory locusts is the transfer of population density information to the next generation via priming during embryonic development. Both egg size and factors released by accessory glands and deposited on eggs during passage through the oviducts or added to the foam covering egg pods are considered crucial for this transgenerational effect (Pener and Simpson, 2009; Tanaka and Maeno, 2010). Cumulative effects on phase characteristics over the entire life cycle are the hallmark of locust polyphenism and have also been found in other groups that exhibit complex life cycle syndromes (Moran, 1994). Thus, elucidating the architecture of the multiple and synergistic developmental switches remains a challenging task for future work.

Aphids, with their highly complex life cycle shifts between sexual and asexual reproduction on the one hand, and winged and wingless morphs on the other, are still an enigma when it comes to mechanisms underlying these shifts, which can occur in different combinations. While crowding is a trigger for the shift from wingless asexual to winged asexual forms, it probably does not involve JH as an endocrine mediator. Juvenile hormone, however, seems to be involved in the photoperiod-induced shift from the winged asexually reproducing form to winged sexuals, a shift that frequently is accompanied by a host plant shift as well (Baker, 1970; Braendle *et al.*, 2006; Le Trionnaire *et al.*, 2008). Genomic analyses have now revealed a surprising alteration in cuticle proteins and the dopamine biosynthetic pathway that may contribute to photoperiod perception and signal transduction involved in this shift (Simon *et al.*, 2010).

In termites, wing development is restricted to primary reproductives, with soldiers and workers (false or true ones) representing wingless phenotypes. Whereas the role of morphogenetic hormones in wing development is still unclear, hormonal regulation of caste development in this clade has been much exploited for pest control, because application of JH analogs can shift the caste-ratio balance toward soldiers (and away from workers and reproductives). Using this well-defined shift as a model, Scharf and colleagues identified hexamerins as hemolymph proteins capable of sequestering circulating JH from exerting its biological effects and showed that the quantitative balance between two hexamerins can be crucial to adjust soldier to worker ratios in a colony according to environmental and social conditions (Scharf *et al.*, 2007; Zhou *et al.*, 2007). The role of hexamerins emerged from differential gene expression screens (Scharf *et al.*, 2003), similar to those performed earlier on genes underlying the formation and function of soldier-specific structures (Miura, 2004; Miura *et al.*, 1999). In this context it will be important to address two sets of questions: (1) the relative immaturity of termite larvae, which, especially in the higher termites, need to be cared for by workers, and (2) the stationary and regressive molts in lower termites, an astonishing phenomenon in insect metamorphosis that emphasizes the importance of molting events in termite societies.

Wing polyphenism in holometabolans takes two very different forms: seasonal wing pattern differences in lepidopterans, and wing reduction in ant workers. Wing pattern polyphenism in butterflies is an allelic polymorphism in camouflage in response to predator pressure, which, in its expression, depends on the timing of the pupal ecdysteroid peak (Brakefield *et al.*, 1998, 2007; Koch *et al.*, 1996). Analysis of candidate genes in wing patterning identified Distal-less as a key factor, and the availability of genomic resources and mutants now sets the road map for deciphering the genomic regulatory network underlying wing eyespot formation (Saenko *et al.*, 2007).

Winglessness in ant workers, in contrast, is an integral element of caste polyphenism in the Formicidae. It is a result of the shutdown of the gene expression cascade regulating wing disc development (Abouheif and Wray, 2002) in response to embryonic/larval hormone titers. While little progress has been made on the endocrine regulation of wing development suppression and worker development in general, a genetic bias to caste fate has now been identified in several ant species (Schwander *et al.*, 2010).

While wing polyphenism is an ancestral character in the caste syndrome of ants, caste polyphenisms in the other social Hymenoptera (wasps and bees) are built on asymmetries in fecundity and, correspondingly, the development of the reproductive system. Such asymmetries are also crucial, and probably were the primary drivers in the evolution of sociality in ants. The focus on *A. mellifera* as a model organism in research on caste development in social Hymenoptera has certainly been promoted by the honey bee's economic importance, a factor that more recently has also stimulated much of the research on the bumblebee *B. terrestris*. Synthesis and titers of morphogenetic hormones have been monitored throughout the entire life cycle of these insects, and these studies have shown that a correlated modulation of JH and ecdysteroid titers drives caste development during the larval stages (Hartfelder and Engels, 1998). Furthermore, the full cascade from initial environmental triggers, through the endocrine response to these, and finally to downstream differentiative processes in target organs, are now starting to be mapped out due to the concentrated efforts in honey bee genomics (Barchuk *et al.*, 2007; Cristino *et al.*, 2006; Evans and Wheeler, 2001). Two emergent factors that may interact with JH and ecdysteroids in different ways are the insulin signaling pathway with TOR as a convergent entry (Azevedo and Hartfelder, 2008; Patel *et al.*, 2007; Wheeler *et al.*, 2006), and epigenetic effects impressively demonstrated through RNAi-mediated knockdown of a DNA methyltransferase (Kucharski *et al.*, 2008).

Another polyphenism related to reproduction, yet going in a completely different direction, is that of male weaponry in dung beetles. The development of these exaggerated morphologies appears to be contingent on at least two critical periods during larval development when horn expression is sensitive to endocrine events. Details of endocrine system functions still need to be worked out, but a great deal of progress has been made in understanding the genetic mechanisms underlying horn growth, which appears to have entailed the co-option of portions of traditional appendage patterning, especially along the proximodistal axis (Emlen *et al.*, 2006, 2007; Moczek *et al.*, 2006).

The past decade has seen an exponential increase in genomic information, not only for classical model organisms in biology, but also for a wide range of species, including those expressing polyphenic traits. Two complete genomes have been sequenced and annotated for polyphenic insect species: the honey bee *A. mellifera* and the pea aphid *A. pisum*. These efforts were crucial for the interpretation of differential gene expression results and candidate gene analyses, and crystallized the following emergent topics: (1) the integration of signaling pathways through expression analyses of nuclear receptors related to the ecdysone and (tentative) JH response and insulin signaling, (2) the role of epigenetic modification through DNA methylation, and (3) the role of small and long noncoding RNAs in developmental regulation. The latter is an emergent topic, especially in human genomics and no doubt will soon influence research underway in developmental biology in most multicellular organisms (Mattick, 2009, 2010).

Species exhibiting phenotypic plasticity, especially the existence of discrete, alternative phenotypes that go well beyond the more common forms of phenotypic plasticity seen in reaction norm variation, are natural experiments on how far genotype-environment interactions can be taken both in ontogeny and in evolution. Many of the polyphenic insects have been established as laboratory populations that can be exposed to different conditions, so that the molecular underpinnings of alternative phenotypes are accessible both at the organismal as well as at the tissue level. This is highly favorable to genome and transcriptome studies, which, without doubt, will come to dominate the field through the advance of new generation sequencing methodologies that will increase sequencing efforts at diminishing costs, not only for DNA and RNA analyses, but also in proteomics. The limits in all these analyses will be set by the advances in bioinformatics tools and computational capacity.

A critical issue to the interpretation of high throughput transcriptome or proteome data is, and will continue to be, the fact that most of the observed molecular level changes are downstream consequences of a "switch mechanism." It may be much more difficult to elucidate genes associated with the switch because there may be only a few genes, and these are harder to detect, and the critical time periods in question occur earlier, before there are obvious morphological differences between the forms. But here polyphenisms excel, because prior endocrine work in most cases has already identified the critical periods when these developmental "decisions" occur and the precise environmental conditions needed to generate animals with each form. Grasping the molecular underpinnings of switch mechanisms will, thus, require carefully devised experiments in combination with high throughput screens. We hope and predict that such approaches will shed light on the ways that hormones (including the elusive JH) alter gene expression to generate distinct alternative plastic phenotypes, and will illuminate the genetic bases of growth, morphology, behavior, and physiology in general (depending on the phenotypic differences relevant to each polyphenic species).

Perhaps the greatest challenge will be to conceptually link the results of such genomic/proteomic studies with organismic-level manifestations in physiology, behavior, and ecology. Here we expect two questions to become predominant. The first one is in the realm of developmental biology and addressing molecular and cellular mechanisms underlying reaction norms and phenotypic plasticity. Many aspects of phenotypic plasticity in insects can be ascribed to general size-related allometries. These have been mathematically formulated in growth models that assume competition for available nutrients between

growing morphogenetic fields (Nijhout and Wheeler, 1994). Such intuitive trade-offs have, however, not yet been taken beyond genetically tractable model organisms, such as *Drosophila*, where gene regulatory networks for life histories and reaction norms are emergent themes (Colombani *et al.*, 2005; Debat *et al.*, 2009; Mirth *et al.*, 2005; Mirth and Riddiford, 2007). The second question is much broader and involves linking evolutionary aspects in the life histories of polyphenic insects to the evolution of regulatory mechanisms underlying the development of alternative phenotypes (West-Eberhard, 2003).

Note added in Proof: A recent landmark publication [KamaKura, M. (2011). Royalactin induces queen differentiation in honey bees. *Nature, Epub,* doi:10.1038/nature10093] convincingly demonstrated that royalactin, an MRJ P1 derived protein that easily degrades in stored royal jelly, is important for queen determination in the honey bee, *Apis mellifera.* It acts through an EGF receptor signaling pathay.

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