The endocrinology and evolution of tropical social wasps: from casteless groups to high societies

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Abstract

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The endocrinology and behavior of three social vespid wasps was studied in northeast Brazil (São Cristóvão, Sergipe) from 2010-2011. There were two main objectives of this work: to test a hypothesis on the origin of reproductive castes (i.e. queen and worker phenotypes) in a communal species (*Zethus miniatus*: Eumeninae), and to describe the endocrinology of two highly eusocial swarm founding species (*Polybia micans* and *Synoeca surinama*: Polistinae). Wasps offer an unparalleled opportunity for research on social evolution due to the continuous range of social organization among extant species. Yet little work has been devoted to the study of wasp physiology beyond *Polistes*, a large genus of primitively eusocial paper wasps. In *Polistes*, juvenile hormone (JH) has been shown to be important for reproduction, dominance, chemical signaling (e.g., cuticular hydrocarbons (CHCs)) in queens while promoting the early onset of certain tasks in workers. Evidence for a similar mechanism in bees and ants suggests a dual function of JH was intact in the last common ancestor of these groups (the sting-

possessing Hymenoptera). The ancestor was undoubtedly a wasp; since eusocial hymenopterans feed helpless brood as they grow, it is likely that the ancestor was also a progressive provisioner, a rare behavior in solitary wasps and bees. The emergence of prolonged maternal care may have set the stage for caste-like phenotypes to arise: an aggressive egg-laying phase (queen-like) followed by an alleged ovary-reduced phase associated with the brood rearing (worker-like). I tested this hypothesis in *Z. miniatus*, a progressive provisioning, group-living wasp with behavioral traits reminiscent of her caste-containing relatives, such as the susceptibility to adopt a hungry larva. Ironically, the high frequency of another behavior characteristic in eusocial societies, the destruction of nestmate eggs, appears to undermine the hypothesis on caste origin: the high rates of brood killing observed in nests of *Z. miniatus* likely explain why females produce multiple eggs at a time (i.e. there is not pronounced ovarian cycle). JH titers did not differ between gravid females looking for a cell (potential brood killers) and provisioners of young larvae. Instead, JH titers were high on a nest where females outnumbered cells, but once cells came to outnumber females, JH levels dropped precipitously, suggesting JH has a role in social competition, a relationship observed in *Polistes*. These results led me to expect a role for JH in reproductive competition in the swarm founders, where multiple, contending queens are the norm. Surprisingly, despite the similarity in the social structure between *P. micans* and *S. surinama*, in which both species determine caste in the adult stage, the endocrine profile of these two wasps could hardly be more different. For instance, queens of *S. surinama* had elevated titers compared to workers whereas JH titers were indistinguishable between queens and workers in oligarchic nests of *P. micans*. In complete contrast to the pattern observed in *Polistes*, only lone queens (lacking direct competition) of *P. micans* had elevated JH in non-swarming nests. JH titers did spike, however, in potential reproductives who were on a nest without a queen. Within a day or two, JH levels plummeted in these prospective replacement queens as they began producing eggs. In *S. surinama*, the rise in JH *followed* behavioral displays of dominance and remained high,

shaping a queen-like CHC profile and likely functioning as a gonadotropin. When these swarm founders shared a pattern in their endocrine profile, they typically contrasted with that observed in *Polistes*. For instance, circulating ecdysteroids are important for establishing dominance in *Polistes*, yet they are not biologically relevant in *P. micans* or *S. surinama*. The wild deviations in endocrine function in these wasps parallel the loss (or inversion) of hormone functions seen in highly derived bees and queenless ants. Fortunately, in the case of wasps, there is promise to track how hormone function was modified through evolution thanks to the preservation of wide-ranging social (and pre-social) arrangements present in today's wasps.

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Chapter 1:
A brief review of social insect endocrinology with an emphasis on the Hymenoptera

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Introduction

The evolution of insect societies has fascinated and challenged biologists ever since Charles Darwin first tried to accommodate for neuter castes in his theory of natural selection (Darwin, 1859). Indeed, sterile workers have attracted much attention over the century for reasons on *why* they forego their own reproduction and help raise another's offspring (Wilson, 1971). The only satisfying explanations to date for the existence of workers are kin selection (Hamilton, 1964a, b), mutualism (West-Eberhard, 1978b) and/or other hypotheses relating to self-interest (e.g., nest-inheritance opportunities among co-nesting non-relatives (Leadbeater et al., 2011)). The resurgence of group selection proponents (Nowak et al., 2010; Wilson and Wilson, 2008), who claim kin selection is inconsequential for the evolution of workers, promises to reignite a debate thought settled decades ago. Whatever consensus is reached on the ultimate reasons for caste evolution, there looms an arguably more important question that addresses the origins of macroevolutionary change: *how*, mechanistically, did castes originate? And once the reproductive castes were established, how were worker and queen phenotypes fine-tuned for a more efficient division of labor?

Worker phenotypes have evolved many times over, from minute sponge-dwelling shrimp (Duffy, 1996) to naked mole rates (O'Riain et al., 2000), but the most well-known examples come from the insects (Wilson, 1971). The ants, honeybees and termites are famous for their amazing emergent complexity and morphologically discrete queens and workers. Except for a handful of highly derived species which employ genetic mechanisms for determining caste (Evison and Hughes, 2011; Hartfelder et al., 2006), to study caste differentiation is to study developmental plasticity, where multiple phenotypes (physiologies, behaviors and morphologies) can be generated from the same genotype. In insects, mechanisms of caste differentiation (and environmental-triggered polyphenisms in general) are typically employed in the pre-imaginal stages (Hartfelder and Emlen, 2011). Juvenile hormone (JH) and the ecdysteroids, master

orchestrators of normal development in all insects (see below), are consistently found to be involved in the bifurcation of developmental trajectories (Hartfelder and Emlen 2011). Indeed, I am not aware of a single developmental polyphenism in insects which has not been shown to rely on prompts from JH or an ecdysteroid. This makes sense given that alternative phenotypes are often nutrition-dependent, and consequently, involve the insulin-signaling pathway which is intimately linked to the endocrinology of the growing animal (Garelli et al., 2012; Mirth and Riddiford, 2007). Before addressing the role of JH and the ecdysteroids in caste ontogeny and function, I will present a brief overview of how these hormones function in normal development.

Juvenile hormones and the ecdysteroids: development and reproduction

Juvenile hormones are sesquiterpenoids synthesized and secreted by the corpora allata (CA), a pair of specialized retrocerebral glands connected to the brain by a nerve which passes through a major neurohemal organ, the corpora cardiaca (Nijhout, 1994). In most insects, JH III is the major JH produced, but lepidopteran larvae produce JH I and JH II and only the adult female produces some JH III (Goodman and Cusson, 2011). Generally, the different JHs have qualitatively similar effects (Goodman and Cusson, 2011 but see Bendena et al., 2011). Analogous to the hypothalamic-pituitary axis in vertebrates, the CA and other endocrine organs are controlled by stimulatory and inhibitory neurohormones derived from the central nervous system (Stay and Tobe, 1978, 1981). In the immature stages, the presence of JH during critical temporal windows of the molting cycle prevents premature metamorphosis (Nijhout, 1994; Riddiford, 1994). Programming for metamorphosis is permitted once JH titers have dropped to undetectable levels, and in most insects studied, ablation of the CA by chemical or surgical means results in a precocious metamorphic molt, and that phenotype is rescued with topical treatments of JH and JH mimics (JHM) such as methoprene (Nijhout, 1994). Ecdysteroids, the

molting hormones, are at the same time inducers of metamorphosis. Indeed, ecdysteroid action depends on the presence or absence of JH (Nijhout, 1994). In most insects, the active ecdysteroid is 20-hydroxyecdysone (20E), although some vegetarian insects, such as honeybees and milkweed bugs, produce Makisterone A instead (the difference relates to using a related plant sterol, rather than cholesterol as a precursor for ecdysteroid biosynthesis) (Feldlaufer et al., 1985; Kelly et al., 1981). For the sake of brevity, I will refer to the active ecdysteroid as 20E when speaking of insects generally.

In adults of most species, JH is essential for ovarian growth, although the control of particular reproductive processes (e.g., vitellogenin synthesis and uptake into oocyte) varies among insect groups (Wyatt and Davey, 1996). Whereas JH continues to be produced by the CA which survives through metamorphosis, the prothoracic glands degenerate and the ovaries assume production of ecdysone, which may be released into the hemolymph (where it is converted into 20E) (Nijhout, 1994). Conjugated, inactive ecdysteroids are loaded in the yolk of eggs where they are later converted into active forms by enzymes to control the embryonic molts (Dorn, 2001). In hemimetabolous insects, such as the cockroach, the CA differentiates around the time of dorsal closure, concomitant with a sudden rise of JH which then declines until hatching (Holbrook et al., 1998). Work on the cricket had shown that JH affects the type of embryonic cuticles made, but in contrast to post-embryonic (i.e., nymphal) development, the presence of JH promotes the production of a precocious cuticles (i.e., advances terminal differentiation) (Erezyilmaz et al., 2004). For example, treatment of JH prior to the second embryonic molt, which normally produces a pronymphal cuticle, shifted the character of cuticle toward a true nymph. This experimentally induced, JH-mediated heterochronic shift in cuticle formation may reflect “a vestige of an ancient developmental mode, and thus could have been reactivated in the line that led to the Holometabola” (Erezyilmaz et al., 2004). In other words, it is hypothesized that an advancement of JH in the embryo of the holometabolous ancestor conspired with 20E to

create the proto-larva, a de-embryonized crawler which preceded the evolution of complete metamorphosis. In support of this, JH treatments to holometabolous embryos have relatively small effects (Truman and Riddiford, 1999, 2002), although high doses can act as ovicides (Staal, 1975).

In addition to orchestrating development and growth, differences in the timing or levels of JH and 20E during the immature stages has been shown to trigger divergent developmental programs (Hartfelder and Emlen, 2011). Below, I will briefly review how JH and 20E can regulate normal growth, yet also have room to direct the production of distinct phenotypes in phylogenetically disjunct lineages of eusocial societies.

Termites

The termites are eusocial cockroaches, being a firm member of Blattoidea (Inward et al., 2007). In terms of discussing phenotypic plasticity, termites are typically divided into two groups: the "lower" (Mastotermitidae, Kalotermitidae and Rhinotermitidae) and "higher" (Termitidae) types (Thorne, 1997). The former are endowed with remarkable plasticity and will be the ones emphasized here. Unlike the holometabolous Hymenoptera and their helpless larvae, the hemimetabolous termites realize their polyphenic diversity in morphology and behavior during the immature stages. In fact, at any given time, there are very few true adults in a termite colony: only the queen and king, and in certain times of the year, winged future reproductives awaiting a signal to disperse, mate and establish a new colony (Korb and Hartfelder, 2008; Thorne, 1997). The worker force consists entirely of "ontogenetically totipotent immatures" (Korb et al., 2012) which exhibit a remarkable degree of plasticity. A hatched larva, defined as a cared-after immature, can realize many possibilities: a nymphal worker, a soldier, a dispersing alate, a neotenic replacement reproductive (non-alate) or even molt indefinitely as a totipotent larva. What's more, in addition to *progressive* (i.e. growth-based) molts, these immature

termites can undergo *stationary* (i.e., no growth) as well as *regressive* molts (Korb and Hartfelder, 2008). For example, in some species, it requires two molts to realize the morphology of a soldier (e.g., big mandibles) or an alate (e.g., wings). If cues which triggered the onset of a developmental trajectory disappear or are inhibited, the individual can revert back to a totipotent form (Korb et al., 2012; Roisin, 1990). Such is the outlandishness of termite plasticity.

Describing the endocrinology of such a plastic, molt-based system is a huge challenge. The literature is extremely fragmented and based on many different species with varying social organizations, making it difficult to piece together a unifying theory on caste differentiation in termites (Korb et al., 2012). Fortunately, there has been recent resurgence of endocrine studies on termites, but the puzzle is far from being solved. Thirty years ago, Nijhout and Wheeler (1982) proposed a model which is still probably the best, skeletonized approach toward thinking about how a single hormone can mediate several developmental switches. In it, they propose three JH-sensitive periods during an intermolt of a totipotent individual: one for sexual characters, one for non-sexual characters and another for soldier characters. Each caste is given a hypothetical (and evidenced-based) pattern of JH titer dynamics throughout the instar. Since its publication, the model has received empirical support, and some authors have proposed additional JH-sensitive periods (Cornette et al., 2008). Other factors, such as instar length, have also been shown to be important, adding to the complexity of caste determination in termites (Korb et al. 2012).

The most studied polyphenism in termites is soldier differentiation, a phenotype which is often realized in two successive molts. In multiple species studied in the field and the laboratory, JH titers are much higher in the presoldier instars, and JH treatment induces soldier formation in workers (Hartfelder and Emlen, 2011), just as the Nijhout-Wheeler model would predict. Soldiers (and even soldier head extracts) have been shown to inhibit soldier development by modifying the transcriptional profile of the soldier development pathway (Tarver et al., 2010),

and a drop or rise in JH in potential soldiers is tightly associated with the worker/soldier ratio (Mao and Henderson, 2010). In a remarkable discovery, Zhou and colleagues (2006) demonstrated that Hexamerin 1 (Hex1), a larval storage protein, covalently binds with free JH in the hemolymph, decreasing its ability to enter cells and be active in physiological processes.. Both nutrition and a suitable worker/soldier ratio positively influence Hex1 accumulation, which physiologically silences circulating JH, thereby preventing soldier development (Scharf et al., 2007).

In general, actively reproducing individuals show a rise in JH titers followed by vitellogenesis, suggesting that JH is a gonadotropin, but the differences of JH profiles during the development of winged reproductives and neotenic replacement reproductives are notable. In order for wings to develop in alates, JH follows the same rules as it does in solitary Hemimetabola: it decreases to a very low level to allow ecdysone to trigger imaginal growth (i.e., to complete metamorphosis). In the case of neotenic development, where wings are not needed, JH spikes in freshly ecdysed nymphs who find themselves in a nest without a reproductive of the same sex (Korb et al., 2012).

The production of alates also depends on extrinsic factors as well, such as rain, since a moist environment is often conducive for nest founding. This predictable seasonal factor led Lanzrein et al. (1985) to suspect that some of the brood were primed for alate development. Interestingly, they found that termite queens transfer both JH and ecdysteroids into their eggs, apparently biasing their ontogenetic progression toward that of a winged adult.

But how did the termites arise from the cockroaches? Termites are thought to be morphologically paedomorphic, retaining juvenile characters throughout their development (being especially evident in the neotenic reproductives), seldom attaining adult cockroach-like features (Nalepa, 2010). Korb et al. (2012), in the spirit of Truman and Riddiford (1999, 2002),

argues that the endocrine profile of termites is paedomorphic as well. She points out that, morphologically, early instar termites resemble cockroaches following dorsal closure. What's more, dorsal closure is shifted toward late embryogenesis in termites, suggesting that late embryogenesis is curtailed compared to that of cockroaches (i.e., "homologously" speaking, termites hatch as 'crawling embryos' or pronymphs). Korb et al. (2012) sees homology in the endocrine profile as well. As mentioned above, JH titers are pronounced following dorsal closure in the cockroach, and titers decrease throughout late embryogenesis and remain low but detectable (and important) in the nymphal stages. In the termite, JH titers are high in early instar larvae, and decline with each successive molt until a developmental switch is made. According to Korb (Korb et al., 2012), "this period, from dorsal closure to hatching of the first nymphal instar in cockroaches, could then be repeated several times in termites, providing flexibility for developmental options". Following this logic, the pronymph of cockroaches is homologous to the "dependent larvae" of termites, and only the final nymphal instar of a termite (when JH levels decrease to allow for imaginal development) is homologous to the nymphs of cockroaches.

The Hymenoptera

The Hymenoptera consist of the sawflies, wasps, ants and bees. This group is famous for their social complexity and assortment of social phenotypes. From relatively simple mother-daughter societies that only occasionally form (e.g., halictid bees) to the amazingly sophisticated metropolises of ants, there is a striking degree of diversity within the Order. As a student, I was astonished to realize that caste-based societies of ants, wasps and bees do not have a common caste-based ancestor. Instead, each lineage has produced queens and workers from solitary antecedents; in the case of wasps and bees, the evolution of castes has occurred multiple times. What predisposes this group for recurrent caste evolution? Historically, much attention

has focused on the peculiar genetic sex determination mechanism of hymenopterans, haplodiploidy, and its role in producing colonies with high genetic relatedness (Hamilton, 1964a, b). Although the perceived contribution of haplodiploidy to kin selection has been seriously questioned (e.g. Hunt, 2007), such discussions on ultimate reasons for worker evolution have dominated hymenopteran research for decades.

More recently, the study of proximate mechanisms in hymenopteran societies has gained momentum. The physiological mechanisms of social insects should not be perceived as passive agents to abstract selective pressures. For example, it is fine to speculate that Mexican blind fish lost sight because vision was not useful and other senses became paramount, but it is quite another to learn that *sonic hedgehog* overexpression in the embryo likely led not only to eye degeneration (by inhibiting *Pax6*), but also caused the jaw size and number of taste buds to increase (Yamamoto et al., 2009), useful attributes for fish living in perpetual dark. The actual biology of organisms is relevant and has constraints in addition to tangible pre-adaptations that allow intangible selective pressures to operate. My thesis work has been an attempt to discover the underlying proximate mechanisms of caste evolution, and to describe how these hypothesized mechanisms were later modified in a long neglected group of advanced caste-based societies. Before articulating the central hypotheses of my theses, a survey of social hymenopteran endocrinology is required.

Caste determination: Development

Like the termites, caste determination in Hymenoptera typically occurs in the immature stages. Unlike the termites, larvae do not participate in colonial maintenance, although the presence of larvae may be essential for other reasons (e.g., protein-rich salivary contributions in *Polistes* (Hunt, 2007) or use as “glue guns” in weaver ants (Wilson, 1971). As holometabolous insects, queens and workers are not realized until the adult stage in the Hymenoptera, and in societies

with morphologically distinguished castes, the construction of alternative phenotypes are necessarily restricted to the pre-imaginal period.

As already emphasized, JH and the ecdysteroids are master regulators of development. As hormones, they have been co-opted numerous times to affect differential morphogenetic events in response to environmental cues, ultimately leading to the production of polyphenisms. Hymenopteran larvae, buffered from the external environment inside their nest, are completely dependent on their adult nest mates for survival. The adults rule the colony and typically control or at least bias the fate of their brood by way of dietary manipulation, although pheromones and mechanical stimuli may also contribute to caste differentiation (see below).

In addition to polyphenic mechanisms in caste differentiation, harvester ants (*Pogonomyrmex*), a genus of stingless (*Melipona*) bees and a swarm founding wasp (*Agelaia*) are suspected to have a polymorphic (i.e., genetic) determination of caste (Linksvayer et al., 2006; Hartfelder et al. 2006; MJ West-Eberhard, *personal communication*). All of these groups are very socially advanced and are clearly derived from ancestors with a polyphenic mode of caste creation (Noll and Wenzel, 2008). How distinct genotypes with a single species respond to endocrine signals has not been explored.

Bees

The most famous and probably best studied case of caste differentiation comes from the honeybees. All early instar honeybee larvae are fed “royal jelly”, a sugary proteinaceous diet loaded with vitamins and antibacterial components, a mixture produced by the hypopharyngeal glands in nurse bees who feed the soliciting larvae. Queen larvae, who occupy specialized rearing cells, receive copious amounts of royal jelly whereas destined worker bees are switched to a diet of honey, pollen and glandular secretions (Winston, 1987). A single protein in the royal jelly, called Royalactin, has been demonstrated to increase body size, ovariole number and

decrease developmental time, all hallmarks of honeybee queens (interestingly, Royalactin has similar effects in *Drosophila*) (Kamakura, 2011). Royalactin also increased the titer of JH during development, a notable result given that JH treatment to otherwise worker-destined larvae induces queen characteristics (Kamakura, 2011). As would be expected based on the feeding protocol, the critical window for JH action occurs later in larval development, and JH titers and CA activity are indeed higher in queens than workers during specific periods in the later instars (Hartfelder, 1990). *In vitro* assays have shown that methoprene stimulates the production of Makisterone A by the prothoracic glands in the cocoon-spinning phase, and application of JH to worker larvae leads to a precocious rise in Makisterone A titers in presumptive queens (Hartfelder and Engels, 1998).

How do elevated JH titers lead to queen characteristics? Among target tissues, the ovary has received the most attention (Hartfelder and Emlen, 2011). Ovariole number of honeybee queens greatly outnumbers that of workers. Instead of a queen-specific proliferation of primordial ovarioles, both workers and queens develop hundreds of ovariole precursors. In workers, the number of ovarioles is then drastically reduced due to a breakdown of the actin cytoskeleton. A single treatment of JH, mimicking the profile of developing queens, prevents this disintegration (Capella and Hartfelder, 2002). Quantitative trait loci mapping identified several genes known to function in apoptosis in *Drosophila* (Linksvayer et al., 2009), some of which may be regulated by JH (Hartfelder and Emlen, 2011).

With the advent of the microarray and transcriptome studies, more and more genes involved in caste differentiation are being identified. Not surprisingly, given the differences in body size between honeybee castes, the target-of-rapamycin (TOR) and insulin-like signaling (IIS) pathways show notable differences in queen and worker development. Although problematic, double stranded RNA interference (dsRNAi) has been shown to be effective in some cases for honeybees. TOR knockdown prevents the development of queen characteristics (Patel et al.,

2007) whereas insulin-like peptides and their receptors are more highly expressed in workers than queens during periods of intensified growth (de Azevedo and Hartfelder, 2008). As will become evident, this is but one example of widely conserved physiological associations which have become upended in the regulation of honeybee ontogeny.

Differential endocrine profiles are also seen in the development of other eusocial bees. As was seen in honeybees, quantitative and temporal differences in JH and/or ecdysteroids, in response to feeding regiments, lead to the production of caste phenotypes (Hartfelder and Emlen, 2011). In stingless bees, an incredibly speciose group of highly advanced eusocial bees confined to the Neotropics, JH application to cocoon-spinning larvae in several species invariably led to queen phenotypes (Hartfelder et al., 2006). Consistently, queen-destined larvae have elevated JH titers in the final instar relative to workers, a period which coincides with differential feeding for queen and worker larvae. In *Bombus*, as observed in honeybees, a queen-specific pre-pupal shift in JH and ecdysteroid titers is seen in bumble bees (Hartfelder et al., 2006). Following the trend seen in the highly eusocial bees, the period of differential feeding in the final larval instar corresponds to a JH-sensitive, caste determining phase in *Bombus hypnorum* (Roseler, 1970). But not all *Bombus* are alike. In *B. terrestris*, the queen pheromone and not nutrition was identified as the critical factor for determining caste. In the absence of the queen pheromone in the early instars, larvae show higher titers not only of JH (Cnaani et al., 2000) but also the ecdysteroids (Strambi et al., 1984).

Wasps

Many wasp species also exhibit clear caste dimorphisms, especially temperate species such as paper wasps (*Polistes*), yellow jackets and hornets. The only objective of these annual colonies is to produce *gynes*, the future reproductives which must survive the winter as diapausing adults, and a larger body affords more energy reserves. The gyne will emerge after the passing of the unfavorable season as a nest *foundress*, intent on establishing a nest or joining other

foundresses who are doing the same. Joining is a strategy which could pay off if she is able to inherit the nest if the queens dies or falters early in the cycle (Hunt, 2007; Leadbeater et al., 2011; Reeve, 1991) (the endocrine-fueled competition among foundresses will be discussed in a later section).

Typically, the first generation (G1) of wasps are female who will become workers unless the queen dies, and the second generation (G2) will be comprised of males and gynes who will mate outside the nest before overwintering. As mentioned, gynes are typically larger than workers, but there is often overlap in body size. Physiologically, however, they are quite different (Hunt, 2007). With limited or (in the case of a lone foundress) no assistance in the raising of G1 offspring, the G1 larvae are provisioned less and consequently develop more slowly than G2 females which receive ample food and are able to develop much more quickly. In addition to nutritive differences, queens from a variety of species of *Polistes* will vibrate or drum G1 larvae more than G2 larvae, and there is experimental evidence that this mechanical stimulus steers the development of G1 larvae toward that of a worker phenotype (Suryanarayanan et al., 2011). Remarkable as this postulated mechanism is, G1 workers can nonetheless take over the nest if the queen dies (i.e., they are primed for reproduction if given an opportunity) (Hunt, 2007; Hunt and Amdam, 2005; West-Eberhard, 1969). Gynes, in contrast, are not primed for immediate reproduction, do not work, have a longer pupal period and are characterized by a distinct physiology which will allow them to diapause (Hunt, 2007). Gynes, like other insects which diapause as adults, may be physiologically committed to diapausing before activating their ovaries (Denlinger et al., 2011). Topical JH application can break the prerequisite to diapause and activate ovarian maturation in *Polistes* (Bohm, 1972; Giray et al., 2005; Hunt, 2007). As adults, gynes are characterized by an up-regulation of Hexamerin 1 (Hex1) and low JH titers (Hunt et al., 2007). This association is eerily similar to that seen in termites, where Hex1 has been shown to bind to and sequester JH so that it cannot be

physiologically active (Zhou et al., 2006). Introduction of *hex2*-dsRNAi to larvae of Polistes resulted in minimal effects, but “trends” toward gyne-like qualities were noted (Hunt et al., 2011). Curiously, studies with *hex1*-dsRNAi were not reported.

The above observations suggested to Hunt (2007) that *Polistes* evolved from a solitary ancestor with a bivoltine (two-generation) life cycle. The ‘diapause ground plan hypothesis’ states that the traits which characterize workers and gynes are very similar to those present in solitary wasps with adult diapause (Hunt et al., 2007). Appealing as this hypothesis is, it assumes a temperate origin of castes in social wasps, whereas all phylogenetic and distributional evidence suggests that social wasps originated in the tropics (Pickett and Carpenter, 2010). Also, the authors ignored that no extant, putative ancestral-like solitary wasp (e.g., eumeninae) undergoes diapause in the adult stage. Instead, when referring to “other insects” which undergo diapause as adults, they cite papers on non-wasps or parasitoid wasps. There is also no consensus that wasp castes originated by means of mother-daughter associations (the ‘subsocial” hypothesis supported by Hunt (2007)) instead of non-related aggregations of adults which eventually became a family group (‘polygynous family’ or ‘semisocial’ hypothesis, a view supported by West-Eberhard (1978b)). The latter is supported by much comparative data, and suggests that monogynous rule is not an ancestral trait but a derived character appropriate for a seasonal environment (West-Eberhard, 1978a). The underlying endocrinology of *Polistes* G1 and G2 development has not been studied. In fact, I am not aware of a single report for JH or ecdysteroid function in the pre-imaginal stages of *any* species of wasp.

There are many highly eusocial tropical wasps (Epiponini) which have independently evolved morphologically distinct castes, and they have evolved in different ways. Sometimes the queens are larger than their workers, and sometimes they differ only is size-independent allometries. The repeated yet unique developmental innovations which led to castes in Epiponini provide a

great opportunity for studying the origins of caste dimorphisms, but little is known about their physiology that goes beyond dissection. Wasps in the Old World (*Ropalidida*, *Polyboides*), which evolved swarming behaviors and caste dimorphisms independent of the New World wasps, offer further prospects for study.

Ants

Studies on complex trait allometry in ant castes are well-known and have been described in mathematical terms (Holldobler and Wilson, 1990), yet investigations on how these phenotypes are actually generated (i.e., pre-imaginal development) are few and mostly rely on JH application experiments. In brief, JH application to brood of various ant genera caused the appearance of queen-like traits or increased the ratio of queens produced (reviewed in Wheeler, 1991). In ant species which produce soldiers in addition to queens and workers (amounting < 5% of total ant species), queen determination occurs very early in development: the embryo (Passera and Suzzoni, 1978; Suzzoni et al., 1980). As was demonstrated in termites, eggs of *Pheidole pallidula* are supplemented with hormones to bias the development of the brood. Whereas JH application to eggs increased the likelihood of queen development (Passera and Suzzoni, 1978) eggs with greater amounts of ecdysteroids typically became workers (Suzzoni et al., 1980). Moreover, queens laying worker-destined eggs had higher levels of circulating ecdysteroids (Suzzoni et al., 1980). Following the completion of embryogenesis and the majority of larval development, another critical window of hormone sensitivity emerges, this time to bifurcate worker vs. soldier differentiation. With the queen and non-queen split already determined in the embryo, methoprene (JHM) treatment to late instar larvae of *Pheidole bicarinata* in this context (cf. ant species with only one worker phenotype) resulted in the development of soldier characters (Wheeler and Nijhout, 1983). Ecdysteroid titers in immature ants have not been measured, but in *Plagiolepis pygmea* the ecdysteroids were higher in the terminal instar of larvae fated to become workers (Suzzoni et al., 1983). A summary of the

fragmentary record of developmental endocrinology in ants indicates that the ecdysteroids are associated with worker development whereas JH is associated with and can induce queen phenotypes (Wheeler, 1991).

Endocrine Regulation of Reproduction and Dominance

Sawflies (Symphyta) are the most primitive hymenopterans and are strictly solitary and mostly herbivorous. The lineage which led to the wasps, bees and ants (Apocrita) likely evolved from within a parasitic lineage of Symphyta, making the group paraphyletic. As in most insects, JH has been shown to function as a gonadotropin in sawflies. Ovaries of females were transplanted to males, and JH treatment of males was shown to induce ovarian maturation (Hatakeyama and Oishi, 1990). Thus, a conserved role for JH in reproduction is preserved in the most basal hymenopterans. But as will be revealed, canonical endocrine functions in adults are often turned upside down in eusocial organisms.

Primitively eusocial bees

In a facultatively eusocial sweat bee, *Megalopta genalis* (Halictidae), mother-daughter association can result in small queen-worker systems (Kapheim et al., 2012). Solitary females result when a daughter disperses and founds her own nest. In all nesting females with developing ovaries, irrespective of the social setting, JH levels (as determined from whole-body extracts and quantified by gas chromatography/mass spectrometry) were low at eclosion and high after 10 days of maturation, coincident with onset of nest founding, suggesting that a gonadotropic role for JH is preserved in *M. genalis* (Smith et al., 2012). In another sweat bee, *Lasioglossum zephyrum*, JH treatment promoted ovary development (Bell, 1973). Solitary reproductives, in contrast, had smaller ovaries and significantly less JH than queens (caveat: ovarian and JH measurements were reported in separate papers and did not come from the same females: ovary data: Kapheim, et al. 2012; JH data: Smith, et al. 2012). These data support a social function for JH, and the authors speculate that JH is important for dominance

interactions in addition to functioning as a gonadotropin. Thus, already in a facultatively eusocial hymenopteran, JH shows an expansion of function to modulate alternative physiologies and behaviors which result from the addition of a social context. A remaining question is whether JH is also modulating pre-imaginal development in response to diet since the mother, a prospective queen, can manipulate the feeding of her daughters and bias them toward becoming workers. Indeed, dispersing females have larger heads than stay-at-home females (i.e., incipient workers), thus implicating JH as having a role in caste formation, although it is not clear if workers are pre-programed to remain on the nest. For example, is the decision to stay or disperse contingent on JH levels during a critical period in adult maturation? Does the presence of the queen, who physically dominates her daughters, suppress JH during this period? Or are factors involved in pre-imaginal development producing distinct physiologies with predictable fates? Obviously, hormone manipulation studies will be needed to begin to assess these hypotheses. Also, the role of ecdysteroids in *M. genalis* has yet to be tested.

The so-called 'primitively eusocial' bumble bees (*Bombus*) represent a distinct transition toward a caste-based system. As with temperate-zone paper wasps of *Polistes*, bumble bees have an annual cycle where the first generation of females are workers. Later in the season, after the 'competition point' is breached, daughters begin to emerge who will challenge the queen for egg-laying rights (Bloch, 1999). Queens are thought to be larger due to the need to diapause through the winter (West-Eberhard, 1978a) but it is sensible to conclude body size is likewise important for maintaining dominance. As already mentioned, the queen releases pheromones to control development and reproduction of workers, but workers retain the ability and sometimes do mature their ovaries in the presence of the queen after the 'competition point' when daughters begin to challenge their mother-queen for reproductive rights (Alaux et al., 2006a; Bloch et al., 2002; Van Doorn and Heringa, 1986). The pheromone appears to be non-volatile, and can induce introduced egg laying workers (taken from a post-competition point colony) to

revert back to sterility (Alaux et al., 2007). Other experiments suggested that the decision is not directly inhibitory on ovarian development. Rather, the signal of reproductive dominance is perceived by “eavesdropping” workers who autoregulate themselves, meaning, it may be in their interest to not challenge the queen (Alaux et al., 2007; Alaux et al., 2006a; Alaux et al., 2006b; West-Eberhard, 1977). Ovipositing workers have elevated JH titers compared to their non-ovipositing worker counterparts, and topical application of JH to non-ovipositing workers can overcome the sterility-inducing effect of the queen pheromone (Roseler, 1977; Roseler and Roseler, 1978).

As would be expected in a colony with reproductive competition, bumble bee colonies are characterized by a dominance hierarchy among workers. Dominance hierarchies also sort out in small groups of queenless workers, with the oldest female typically becoming the alpha female. Subordinate, physically dominated females possess small ovaries and have reduced CA activity compared to their aggressors (Van Doorn, 1987). Therefore, in all contexts, ovarian maturation is inhibited by signals (e.g., pheromones or aggression) which ultimately lead to reduced CA activity. Removal of the queen results in a change of JH biosynthesis within a day followed by a rise in JH titers in prospective replacements (Bloch et al., 2000a; Bloch et al., 2002; Shpigler et al., 2012). The above results would seem to suggest a role for JH in dominance, but a surge in JH titers occurs only *after* the dominance hierarchy is established, and treatment with JH did not increase worker dominance in any social condition (Bloch et al., 2000a; Bloch et al., 2002). Thus, JH is not important for aggression per se.

Following the rise in JH titer, females with maturing ovaries experience an upsurge in circulating ecdysteroids, followed in turn by a detectable lengthening of the oocyte (Bloch et al., 2000b). Hemolymph ecdysteroids are highly variable, but in general queens have more than workers, and queens heading a colony, who have active and larger ovaries, tend to have more hemolymph ecdysteroids than virgin, mated pre- and post diapausing queens (Geva et al.,

2005). A pronounced difference in ovarian ecdysteroids among workers was seen as oocytes begin incorporating vitellogenin. Ecdysteroids are associated with dominance, but are not important for the onset or maintenance of dominance (Geva et al., 2005). Ecdysteroid titers are low when the social hierarchy is being established (Bloch et al., 2000b) and ovariectomy has no effect on dominance status (Van Doorn, 1987, 1989). Factors such as brood presence, brood composition and nest/group demographics also influenced ecdysteroid levels (Geva et al., 2005).

Thus, in bumbles bees, JH is gonadotropic in a variety of social contexts and is associated but not necessary for dominance behaviors. The role of ecdysteroids is more enigmatic. Might these hormones also influence the cuticular hydrocarbon (CHC) profile or signaling of emerging reproductives, as has been implicated for JH in wasps (Izzo et al., 2010) and 20E in house flies (Blomquist et al., 1984)? To my knowledge, this possibility has not been explored.

Primitively eusocial wasps

In another lineage of primitively eusocial hymenopterans, *Polistes*, JH and the ecdysteroids are also associated with dominance and reproduction. In contrast to *Bombus*, treatment studies have shown that JH and ecdysteroids are genuine drives of aggression in certain contexts, such as nest-founding (Roseler, 1985; Roseler et al., 1984; Tibbetts et al., 2011; Tibbetts and Izzo, 2009). Historically, endocrine studies have focused mostly on dominance battles between springtime foundresses where the most dominant will become the queen. Throughout the 1980s, the laboratory of Roseler demonstrated that 1) winners have larger CA and higher JH activity (Roseler et al., 1980, 1984), 2) JH synthesis is positively correlated with ovarian development (Roseler et al., 1984, 1986), 3) high JH titers and ovarian maturation only persist in winners (i.e., queens) (Roseler and Roseler, 1989; Roseler et al., 1984, 1986) and, among other findings, 5) JH treatment increases the odds of a victory, providing a significant competitive boost to foundresses who possess small CA (Roseler et al., 1984; Strambi, 1990).

Also, foundresses injected with 20E showed an increase in dominance (Strambi, 1990). Notably, JH plus ecdysteroid treatment did not increase the odds of victory more than 20E injection alone (Strambi, 1990). However, the story is not so simple. Some ovariectomized (i.e., ecdysteroid-less) foundresses who are established dominants can maintain their position as the alpha female. Although those who remained dominant typically had larger CA, there were instances where the ovariectomized females were supplanted by co-foundresses with smaller CA (Roseler and Roseler, 1989). Even among competing foundresses, JH and facial markings (see below) are better predictors of dominance than ovary size (Tibbetts et al., 2011). After the hierarchy settles, queen ovaries will mature while subordinate ovaries begin to regress (West-Eberhard, 1969).

In *P. dominulus* (along with 5 other independently derived lineages within the genus), facial patterns convey honest signals of agonistic ability and are thought to minimize the risk of direct physical tests of dominance with rivals (as seen in other *Polistes* species where fights can be fatal) (Cervo et al., 2008; Tibbetts, 2002). Remarkably, foundresses of *P. dominulus* are able to use each other's facial patterns to assess the fighting ability of other foundresses before risking an interaction (Sheehan and Tibbetts, 2010; Tibbetts et al., 2010). Facial patterns are particularly important for establishing dominance early in the nest founding phase, when foundresses are interacting for the first time. Over the next days or weeks of co-existence, the patterns become less important as actual agonistic ability is assessed, and indeed, when facial pattern failed to explain dominance, JH titers did (Tibbetts and Izzo, 2009; Tibbetts and Sheehan, 2012). As the colony continues to stabilize, facial patterns may become irrelevant, with signaling now conveyed through CHCs on the epicuticle of the queen. JH is a gonadotropin in *Polistes* (Giray et al., 2005; Roseler et al., 1984; Tibbetts and Sheehan, 2012), but JH titers show an even stronger correlation with CHC fertility signaling in *P. dominulus* (Izzo et al., 2010). Whereas JH and facial spots correlate with dominance, ovary size and body size consistently

fail to do so early in the nesting cycle. Despite this, a positive correlation between JH titers (or CA volume/activity) and ovary size is usually present (Tibbetts et al., 2011).

In stable colonies of *Polistes*, one queen rules and is usually uncontested, although there is a linear dominance hierarchy beneath her (West-Eberhard, 1969). In normal queenright colonies, queens tend to have higher JH titers than workers, and JH titers do not correlate with aggression in workers. If the queen is removed, JH titers soar in workers who initiate aggressive acts. During this period of instability, one of the aggressive, JH-fueled females will become the next queen (Tibbetts and Huang, 2010). The context-dependent relationships between JH, aggression, dominance and competition strongly resemble patterns of testosterone documented during periods of competition in birds and other mammals. The empirically supported 'challenge hypothesis' predicts that testosterone titers will increase in periods of male-male competition, providing a boost to an individual's fertility and mating success but at the expense of a compromised immune system and greater mortality (Roberts et al., 2004; Wingfield et al., 1990). In the case of female-female competition in wasps, the costs of high JH may include reduced immunity, although no direct test for this has been carried out in social wasps. Concerted attempts to apply a JH-based 'challenge hypothesis' to other insect taxa, such as the burying beetle (Scott, 2006) and lobster cockroach (Kou et al., 2008), have failed despite a positive correlation between JH titers and aggression in these groups. As with the bumble bees, a closer look reveals that JH may not be high at the onset of the fights, or the data is inconsistent (Trumbo, 2007). To my knowledge, the direct link between JH and dominance is specific to *Polistes*. It will be interesting to see if this ground plan is intact in more as well as less derived species of social wasps.

Derived eusocial Hymenoptera

In three independently derived lineages of advanced eusocial hymenopterans, the ants, the stingless bee and honeybees, JH has lost much if not all of its gonadotropic functions in the

adult (Hartfelder and Emlen, 2011). In the adults of honeybees and a species of stingless bees (*Melipona quadrifasciata*), JH has no apparent function, as demonstrated by low titers in both species (see Hartfelder & Engel, 1998). Moreover, neither JH treatment nor allatectomy has any effect on honeybee queens (Ramamurty and Engels, 1977). In honeybee queens (and workers), a gonadotropic function for JH has been shifted to the pupal stage, where it stimulates vitellogenin synthesis and then declines (Barchuk et al., 2002). Ecdysteroid treatment in the pupal stage, on the other hand, retards the appearance vitellogenin (Barchuk et al., 2002). In both honeybees and singles bees, hemolymph ecdysteroid titer levels were indistinguishable between the queens (including virgins vs. active egg layers) and workers. In fact, ecdysteroid titers were undetectable in queens of stingless bees (Hartfelder et al., 2002).

In Ponerine ants, hormonal regulation of reproduction has also been lost or become inverted with respect to reproduction in two species (*Streblognathus peetersi* and *Harpegnathos saltator*) of secondarily queenless ants. In these societies, dominant workers (called gamergates) express themselves with outright aggression and chemical signaling. Application of pyriproxyfen, a long-lasting JHA, to alpha females reliably caused a decrease of vitellogenin in the hemolymph and demotion in social rank which was accompanied by a shift in CHC profile toward that of a non-reproductive (Cuvillier-Hot et al., 2004). In a closely related species which has retained the queen caste, JH and ecdysteroid titers do not differ among queens, gamergates and inside workers, indicating that the loss of JH as a gonadotropin was not a consequence of losing the queen (Penick et al., 2011).

Interestingly, JH does function as a gonadotropin in the more highly derived fire ants (*Solenopsis*), but it is unclear if this function is retained from the common wasp-like ancestor of ants, or was lost (cf. basal ants) and regained. In brief, JH levels are high in queens who pheromonally inhibit the dealation (wing-shedding) of future reproductives by suppressing JH biosynthesis (Brent and Vargo, 2003). Fortunately for myrmecologists, the use of precocene, a

plant-derived cytotoxic agent which targets and destroys cells of the CA, has been shown to be effective in *Solenopsis*, an exceptional case for holometabolous insects. Precocene application prevents the shedding of wings in queenless alates, and JH application rescues dealation (Burns et al., 2002).

Nothing is known about JH or ecdysteroid function in the adult stage of advanced societies of wasps. In some societies of Epiponini swarm founding wasps, newly eclosed individuals have the capacity to become a queen or worker, and their physiological development depends on the social context (West-Eberhard, 1977). In these species, rather than producing morphologically specialized phenotypes (as observed in giant egg layers of termites and ants, for example), evolution has prioritized caste flexibility due to the constant threat of nest destruction or invasions (e.g., by raiding ants) (Jeanne, 1991). If the colony is forced to abandon their nest, they can build a new one within days and queens can be replaced by the most dominant workers. The lack of caste dimorphism of many epiponine wasps has led some authors to conclude, I believe erroneously, that the common ancestor of Epiponini, like that of some Ponerine ants, were queenless (Noll and Wenzel, 2008). This hypothesis is not supported because polygyny (multiple queens) is a common feature of tropical wasps, while long-term monogyny (e.g., the well-studied species of *Polistes*) is a derived, temperate-zone adaptation (i.e., swarm founders did not evolve from a monogynous ancestors) (West-Eberhard, 1978a). However, queen numbers do fluctuate in colonies of at least some (if not most) epiponine wasps (West-Eberhard, 1978, 1981), with colony rule alternating between polygynous and monogynous phases. Termed ‘cyclical oligogyny’, this peculiar strategy probably explains the high relatedness among workers despite a high variance in queen number (Hughes et al., 1993). Dominance in these wasps are typically expressed with ritualized act of aggression, displays which communicate honest signals of fighting ability (performed by queens) or screen for weakness in queens (performed by workers) (West-Eberhard, 1977, 1978b). Thus, by

studying the adults in monomorphic swarm founding societies, one can simultaneously investigate the endocrine underpinnings of caste determination, dominance signaling, queen reproduction, queenless reproduction, and worker behaviors. The studies presented in Chapter 3 and 4 are the first attempt to elucidate the hormonal basis of the caste-based life in Epiponini.

Endocrine Regulation of Worker Behavior

For a long time, the loss of a gonadotropic role for JH in highly eusocial hymenopterans appeared to have been swapped for a novel function in workers. In honeybees, it is well established that JH modulates age-related transitions in tasks (i.e., temporal polyethism) (Robinson and Vargo, 1997). As in many hymenopteran societies which do not possess morphologically distinguished workers (e.g., higher ants, bumble bees), queenright honeybee workers begin performing in-nest tasks (e.g., nursing brood) within the first few days of adult life. After 2-3 weeks, they transition to off-nests task, meaning, they begin to forage (Winston, 1987). This behavioral conversion is marked by a pronounced rise in JH titer which remains elevated; but if nurse bees are removed, foragers are able to revert to nursing which is concomitant with a decrease in JH titer (Robinson and Vargo, 1997). The fact that JH is modulatory and not essential for foraging was demonstrated by allatectomy experiments which only delayed the transition to foraging. Also, JH or JHM application to nurse bees induces precocious foraging (Sullivan et al., 2000). Until 1993, this novel function for JH was thought to be an idiosyncrasy of honeybee physiology. In the only hormone study to date on any highly eusocial wasp, O'Donnell and Jeanne (1993) showed that methoprene treatment to young queenright females of *Polybia occidentalis* led to a dose-dependent advancement in behaviors (e.g., appearance on envelope, nest maintenance, foraging, etc.). Although titers were not measured, it provided evidence that JH regulates temporal polyethism in two separate lineages of eusocial Hymenoptera, and the authors concluded, reasonably, that JH independently evolved worker-specific functions in bees and wasps.

West-Eberhard (1996) disagreed. In a book chapter which would come to inspire the present Thesis, she hypothesized that the parallel functions of JH in regulating age-related changes in behavior in phylogenetically disjunct species of eusocial Hymenoptera is actually a conserved mechanism which could be found in other social Hymenoptera with temporal polyethism and, necessarily, in the ancestral-like non-social wasp (ants and bees, evolutionarily speaking, are simply derived wasps) (West-Eberhard, 1987; 1996). She further postulated that a cycle of JH biosynthesis is superimposed on the behavioral and putative ovarian cycle of progressive provisioning wasps. In one phase, JH acts a gonadotropin. In the subsequent provisioning phase, when the ovaries are reduced or at least quiescent, JH assumes foraging-related functions.

Although aspects of West-Eberhard's hypothesis about the origin castes have been questioned (see Chapter 2), subsequent work has shown that a modulatory function of JH in workers is indeed widespread in the eusocial Hymenoptera, making the split-function hypothesis more parsimonious than a scenario which espouses multiple independent origin events. In *Polistes dominulus*, where foraging is an age-related task, methoprene application induces precocious foraging (Shorter and Tibbetts, 2009) although foragers have not yet been reported to have high JH titers (Tibbetts and Huang, 2010). In *Polistes canadensis*, where guarding is an age-related task, JH titers are higher in guards and the JHM methoprene induced precocious attacking behavior and also boosted the proportion of foragers (Giray et al., 2005). Taken together, these results suggest that JH is involved in modulating age-related transitions in task. In ants, the evidence is much stronger. In Ponerine ants, as mentioned above, JH levels are not distinguished between queens, gamergates and in-nest worker, yet foraging workers have elevated levels of JH (and lower ecdysteroids) (Penick et al., 2011). In harvester ants, JH titers were higher in foragers than nurses in both age-typical and single-cohort (single-age) colonies (i.e., JH was the principal correlate of foraging activity) (Dolezal et al., 2012). An elevation of JH

titer is also seen in dispersing, nest-founding queens of this species (Dolezal et al., 2009). Thus, a non-reproductive function for JH appears to have deep roots and has even been co-opted for queen behaviors.

A final note must be made about the regulation of JH titer in worker honeybees. As mentioned above, JH stimulates vitellogenin synthesis in the pupa of both queens and workers (Barchuk et al., 2002). At eclosion, however, the relationship is flipped: vitellogenin is high and inhibits JH synthesis during the nurse phase. As a nurse transitions into a forager, viteollgenin drops slightly and JH titers now begin to suppress vitellogenin (Amdam and Omholt, 2003). Consistent with this scenario, RNAi for *vitellogenin* led to precocious foraging (Guidugli et al., 2005). In honeybees, vitellogenin is a major zinc transporting protein shown to directly affect immunosenescence of hemocytes (Amdam et al., 2005) which corroborates data that nurses are more resistant to stress than older workers (Remolina et al., 2007). This innovative physiological module of caste regulation, first predicted by modeling studies (Amdam and Omholt, 2003), is thought to have been co-opted from a reworking of an ancient reproductive ground plan, although hypotheses regarding *how* this occurred remain vague.

Major gaps in our understanding of social evolution

The present Thesis aims to fill major holes in our current understanding of proximate mechanisms of eusocial evolution. Much of the literature on eusocial insect evolution alludes to ‘reprogramming’ or ‘co-option’ of a solitary insect ground plan for the creation of novel physiological/behavioral mechanisms of caste regulation observed in eusocial societies. Citations frequently lead to articles describing the physiology of *Drosophila*, one of the most highly derived insects on the planet and thus not an appropriate model for what the ancestor may have been like. With emerging developmental and phylogenetic evidence that larval termites are literally the crawling embryos of cockroaches, significant insights into caste

evolution lie ahead. In the Hymenoptera, where castes have evolved around 9 times, tracking down the most relevant ancestor is more problematic. The beauty of West-Ebehard's hypothesis (1996) on the origin of castes is that it postulates that the deep physiological ground plan of Hymenoptera is, incidentally, ready-made for caste creation when certain characters arise. As will be fully addressed in Chapter 2, the emergence of progressive provisioning, whereby brood are fed as they grow rather than receiving all their provisions at once, may be a major preadaptation for caste creation. All ants, honeybees, most bumble bees and all eusocial vespid wasps feed their larvae progressively, whereas most solitary (non-parasitoid) wasps and bees are mass provisioners. And because ants and bees are derived from wasps, the best extant approximation for the common ancestor to eusocial lineages with progressive provisioning would be a tropical solitary or group-living wasp who progressively provisions her larvae. West-Eberhard (1987, 1996) identified *Zethus miniatus* as an ideal candidate for studying proximate mechanism of evolution but little work has been published on this species since. Thus, my first objective was to find this obscure wasp and test West-Eberhard's long-standing hypothesis on the origin of castes by describing the endocrinology and behavior of *Zethus miniatus*. The null hypothesis: JH titers do not show a significant difference with respect to the behavioral and ovarian cycle (reproductive vs. foraging phase). As will be revealed, the lack of a pronounced ovarian cycle in *Z. miniatus* undermined the hypothesis, and launched the research in a different direction: brood killing and potential role of JH in aggression in a pre-eusocial wasp.

At the other social extreme, we know nothing about the endocrinology of highly social wasps. Not only would a description of hormone profiles in highly eusocial provide an independent test of whether gonadotropic hormones lose their function with increasing levels of social evolution, but it would provide an opportunity for tracking how endocrine patterns change with evolution. As opposed to ants, which are all eusocial (e.g., solitary ants do not exist) and bees, which have

a much more fragmented or less continuous representation of social systems (e.g., there is no primitive honeybee), wasps cover the entire spectrum from solitary species to colonies with millions of individuals and morphologically discrete castes which are suspected to be genetically determined. Thus, for any innovation discovered in the social evolution of wasps, we may be able to study how the mechanism was realized. In Chapters 3 and 4, I present my attempts to dissect the endocrinology of two highly eusocial genera of wasps which postpone queen and worker differentiation until the adult stage, permitting the simultaneous evaluation of caste determination and mechanisms regulating caste physiology and behavior. Given the roles of JH and ecdysteroids in dominance, reproduction and fertility signaling in competing foundresses and prospective reproductives of *Polistes*, I expected these functions to be intact in the highly eusocial wasps.

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Chapter 2:
The endocrinology and behavior of *Zethus miniatus*

Table of Contents

Introduction

The emergence of caste-based animal societies marks one of the greatest events in the history of life on Earth. Some of the more spectacular examples are seen in the sting-possessing wasps, bees and ants (Hymenoptera: Apocrita: Aculeate), each of which has attained, from distinct solitary antecedents, a colonial populace rivaling urban city populations (Zucchi et al., 1995). Tremendous gains have been made in our understanding of societal evolution in all three groups, and research on these phylogenetically disjunct societies support the hypothesis that queen and worker phenotypes emerged by way of pre-existing endocrine mechanisms operating in solitary ancestors (Hartfelder and Emlen, 2011). Yet, as many authors have lamented [see for example (Evans, 1958) and (Wcislo and Tierney, 2009)], the paucity of physiological data on species with incipient, facultative or supposed ancestral-like social traits has made it difficult to assess proximate level hypotheses on the emergence of queens and workers. Recent reports on the physiology and endocrinology of a facultatively eusocial sweat

bee, *Megalopta genalis*, are thus welcomed strides of progress on the question of caste origins (Kapheim et al., 2012; Smith et al., 2012)

In addition to bees, the study of wasps provides researchers with a great opportunity for tracking caste evolution, especially for lineages which have attained high levels of social complexity. Nearly all conceivable levels of sociality can be found within extant wasp species (West-Eberhard, 1978) (Hunt, 2007; West-Eberhard, 1978), and even the ants and bees themselves originated from solitary wasp ancestors. Moreover, there are genera of solitary nest-building wasps which span the spectrum in terms of maternal care (Roubaud, 1910), featuring species which nurture their brood throughout the larval period, an adaptation which characterizes all eusocial vespid wasps, ants, allodapine bees, bumble bees and honeybees (Field, 2005). This strategy, called progressive provisioning, is rare among solitary bees and wasps which, by contrast, are usually mass provisioners – they stock the cell with an abundance of food prior to oviposition. One advantage of the vespid wasps for research on the origins of castes is that they often occupy aerial, open nests where the behavior of adults is in full view, in contrast to bees, which nest in burrows in the ground or in twigs so that observation of social interactions with brood or among adults in nature is difficult or impossible.

The switch from little to extensive maternal care may have created nascent phenotypes with endocrine and ovarian correlates conducive to the evolution of castes (West-Eberhard, 1996) evidenced by the fact that at least three independently derived eusocial Hymenoptera (wasps, ants and bees) employ similar endocrine mechanisms for regulating caste ontogeny and behavior (Dolezal et al., 2012; O'Donnell and Jeanne, 1993; Penick et al., 2011; Robinson and Vargo, 1997; Shorter and Tibbetts, 2009). Specifically, the sesquiterpenoid juvenile hormone (JH) regulates reproduction and aggression in queens (except in some highly derived societies) while reportedly modulating age-related behavioral transitions of workers (Hartfelder and Emlen,

2011). Parsimony suggests that the dual functions of JH were intact in some form in the solitary ancestor of eusocial Hymenoptera (Giray et al., 2005; West-Eberhard, 1996).

The transition to progressive provisioning also results in longer periods between ovipositions. Since aggression is associated with egg-laying among solitary wasps that nest in close proximity (Wcislo et al., 1988), two nascent phenotypes are realized: a *cell building, aggressive and egg-laying* phase followed by a *brood guarding, foraging, and ovary depleted* phase (West-Eberhard, 1987a). The Ovarian Ground Plan hypothesis regards these temporal phenotypes as the substrate from which queens and workers, respectively, emerged. And just as JH dually regulates both queen and worker function across independent lineages of Hymenoptera, the Ovarian Ground Plan predicts that JH is involved in the cyclical changes of ovarian growth and behavior in progressive provisioning species (West-Eberhard, 1996).

A common physiological gound plan to both solitary and eusocial wasps may also be revealed by determining JH titers throughout the ovarian and behavioral maturation of young females (West-Eberhard, 1996). For instance, the well conserved sequence of tasks performed by monomorphic workers in hymenopteran societies is preserved in solitary or sub-eusocial females: nesting activities followed by foraging. JH has been implicated to be important for cell construction in a number of eusocial wasps and bees (Roseler et al., 1985; Shpigler et al., 2012) including both queens and solitary foundresses of *M. genalis* (Smith et al. 2012).

The Ovarian Ground Plan was articulated based on the ovary of a solitary progressive provisioner, *Bembicinus japonicus* Iwata (1955) (see West-Eberhard 1996 p. 294), supplemented by observations of a group-living, casteless wasp: *Zethus miniatus* (Figure 1) (West-Eberhard, 1987a). *Z. miniatus* is one of three known ‘primitively social’ vespid wasps (Eumeninae), all of which progressively provision (West-Eberhard, 1987a; West-Eberhard, 1987b; West-Eberhard, 2005). In addition to feeding and protecting their own larvae as they

grow, females engage in a “proliferation of alternative behavior patterns not observed in solitary wasps” (West-Eberhard, 1987a). Instead of building a cell, gravid females can reuse or usurp a cell of another, sometimes killing young brood in the process (cf. queens of eusocial wasps). Rather than relying exclusively on hunted prey, a mother can rob provisions from neighboring cells. If a female does not wish to remain on her natal nest, she may initiate a nest elsewhere. And perhaps most notably, broodless females, by way of their maternal instincts, are known to adopt orphaned larvae (cf. workers of eusocial wasps, although there is no record of a female caring for more than one larva at a time). Such a range of behaviors could, ostensibly, lead to dominant egg layers and subordinate, non-reproductive care-takers (see West-Eberhard, 1987a, 2003).

Aside from taxonomic descriptions, all previous work on *Z. miniatus* was strictly ethological (West-Eberhard, 1987a), and dissections were not performed, owing to the observation value of every female on a few scattered nests. Therefore, the existence of a pronounced gonadotropic cycle was not investigated in *Z. miniatus*, although progressive provisioning wasps, in general, have much reduced ovaries (Evans, 1966; Iwata, 1955; O'Neill, 2001). In other insects which develop one oocyte (*Glossina austeni*) or ootheca (*Diploptera punctata*) at a time, measurements of corpora allata size or activity strongly suggest a pronounced rise and fall of JH during ovarian maturation which is essential for normal development (Stay and Tobe, 1978; Tobe and Stay, 1977). In turn, ovarian growth is important for regulating JH biosynthesis (Stay and Tobe, 1981). Might a similar mechanism have evolved in progressive provisioning wasps, given their purported protracted period between egg lays? I report here that female *Zethus miniatus* have oocytes showing a greater range of development than that proposed by West-Eberhard (West-Eberhard, 1987a) to represent the fundamental Ovarian Ground Plan. I also show that JH titers do not fluctuate according to cyclical and maturational states of the *Z. miniatus* females.

Methods

Wasps

All behavioral and physiological data were collected from *Zethus miniatus* de Saussure (1875) [Hymenoptera: Vespidae: Eumeninae].

Field studies

Field work was carried out on the campus of Universidade de Federal Sergipe (UFS), São Cristóvão, Sergipe, Brazil. Each nest discovered had been founded on wire hangers or plastic mesh fastened to the walls or eaves of buildings. The entire population was likely descended from a single nest discovered at UFS in 2009 (Fabio S. Nascimento, pers. comm.). All nests were studied *in situ*.

Females were collected within the first couple days of observation (or as they appeared), anesthetized by exposure to low temperature (0 ° C) and marked with oil-based paint pens (Sharpie) on the thorax. As the wasp awoke, she was released in close proximity to the nest. Recently eclosed females, who may have been too inexperienced to know the local environment, were placed directly onto the nest. Male identity was not tracked.

Although several nests in the area were monitored from 2010-2011, the vast majority of observations were carried out on two focal nests large enough to allow for periodic sacrifices, amounting to ~110 hours of observation over 67 days. Observation periods were concluded when the owners and contents of all (or most) cells were determined. Both nests consisted of roughly 40 cells, consisting of 15-25 open cells and 15-25 adult females at a given time.

Nest A (Figure 1) was studied from January 24th to March 4th, 2010 and Nest B from October 31st to December 7th, 2011. On most days, the nests were observed until all adults were surveyed, cell owners identified and cell contents described (amounting to 1.5-2 h of

observation per day). The latter task required a small but strong flashlight because the eggs are laid deep in the cell).

Laboratory analyses

Procedure for collecting haemolymph, cuticular hydrocarbons and ovary measurements:

Females were removed from the nest and immediately put on ice. After ~20 minutes, the anesthetized wasp was removed and pinned to a substrate with intersecting insect pins. Using a 5 µl graduated microcapillary (Drummond Scientific Company) pulled to a point over a flame, 0.75 to 1.3 µl of haemolymph was withdrawn from between the anterior-most segments of the gaster. Each sample was transferred into 500 µl of acetonitrile inside a 2 ml screw-top glass vial capped with a Teflon-lined rubber septum. These samples, each representing a single individual, were used to measure JH titers by radioimmunoassay (RIA). Hormone samples were kept at -20ºC until transport. Ovaries were carefully removed in cold E&B Ringer's solution, photographed with a Leica EZ4D Microscope Camera and were measured using Leica Application Suite software (Leica Microsystems).

All hormone samples were transported on ice to the Universidade de São Paulo, Ribeirão Preto, where they were processed.

Juvenile hormone titer analysis by radioimmunoassay (RIA)

JH was extracted from the haemolymph following a liquid-phase separation protocol developed for honeybees (Huang et al., 1994). The acetonitrile extract was combined with 1 ml 0.9% NaCl and 1 ml hexane in a 5 ml glass tube. After a thorough vortexing, the phases were allowed to separate on ice for 10 minutes, followed by centrifugation at 700 *g*. The supernatant hexane phase, containing the JH, was transferred to a new tube and the hexane extraction was repeated twice. The pooled hexane phases were dried by vacuum centrifugation and the

extraction residues were redissolved in 50 µl toluene (0.5% propanediol) and transferred to RIA glass vials. Just prior to the RIA, the solvent was removed by vacuum centrifugation.

In preparation for the RIA, the JH-specific antiserum was diluted 1:1250 in phosphate buffer containing 0.1% bovine serum albumin and 0.1% rabbit immunoglobulin G. As a tracer, we used [10-^{3}H(N)]-JH III (spec. activity 19.4 Ci/nmol, Perkin Elmer Life Sciences, Waltham, MA, USA), diluted in 0.1 M phosphate buffer (pH 7.2-7.4) (made from 0.2 M mono- and dibasic potassium phosphate stock solutions and 0.02% sodium azide) to 6000-6500 cpm/50 ml. Synthetic JH III (Fluka, Munich, Germany), the lone species of JH known in Hymenoptera (Goodman and Cusson, 2011), was used as a non-radioactive ligand. Two standard curve replicates were set up to cover a range of 25 pg – 5 ng.

The RIA was performed according to the protocol devised by Goodman *et al*. (Goodman et al., 1990). After an overnight incubation at 4ºC, saturated ammonium sulfate was added (50% final concentration) to precipitate the antibody-bound JH-III. Standard curve values were log/logit transformed, and a linear regression was produced to determine JH-III equivalents (pg/ml haemolymph) (i.e., titers) for each sample.

Results

Behavior

Males

Individual males were seen on several nests in the area. Males attempted to mount unmarked, putatively virgin females. Courted females were inferred to have eclosed on the same or previous day, but exact ages were not known. On one occasion, a pair flew off the nest together and mating was observed in the surrounding environs (Lucas Oliveira, pers. comm.). Males were never observed to court females engaged in maternal behaviors.

Male-male aggression on the nest was common. Immediately after contact, males would lunge toward one another with open mandibles. If neither male retreated from the nest, a grappling match ensued, and both males tumbled off the nest. Typically, after 1-3 rounds of this, only one male would return. Male vigilance involved turning toward any movements on or near the nest, and in several instances, their pursuit led to the departure of resting chalcidoid wasps from peripheral perches overlooking the nest. Females were observed to attack males but not vice versa. Males would often rest inside cells containing young brood while the mother was foraging. Upon return, the mother would attempt (usually successfully) to drag the male out by an antenna and often lost prey in the process.

Females

I. Young (recently eclosed) females

Very young females often disappeared from their natal nest for several days before returning to initiate maternal behaviors; some never returned. A female's first cell was either newly built by her, or a re-used empty cell built by another female. I never observed a female who had not yet laid an egg usurp a cell and kill the brood of another.

II. Mated females who had oviposted and exhibited maternal care ('mature' females)

Usurpation and offspring mortality

In the population studied, cell re-use was the preferred means of cell acquisition. In cases where there were no vacant cells, gravid females would often attempt to usurp the cell of a nestmate rather than construct one from scratch. These aggressive, cell-seeking females refrained from taking over cells containing large larvae but eggs and small larvae were often destroyed and tossed off the nest if left unguarded (curiously, the proteinaceous young were not eaten). The usurper would then "claim" the cell by ensconcing abdomen-first into the cell, awaiting the arrival and inevitable challenge by the unwitting mother.

When a female recognizes that her cell is occupied by another, she usually attempts to bite and drag the interloper out of the cell. Such interactions could go back and forth for an hour, at which point the loser may attempt to procure the cell of another or become idle until she begins to seek a cell for oviposition. Three females whose cells had been usurped became very alert patrollers and defenders of the nest, a “team task” which benefits the entire colony. In one instance, a patrolling female without a cell repeatedly attacked a foreign, unidentified wasp that approached the nest. At other times, these vigilant females chased away invading chalcidoid wasps.

Offspring mortality was quantified on two nests from the same population at different times of the year. In Nest A, only 51% of eggs reached the spinning prepupal stage (n = 71 eggs from 32 females) whereas offspring from Nest B enjoyed a 75% success rate (n = 57 eggs from 27 females) (Figure 2). It should be noted that these percentages are overestimates since eggs could be laid and disappear between daily surveys (which was suspected though not scored in several cases).

In both nests, only eggs and young to middle age larvae vanished (Figure 2). The killing of offspring by nestmates attempting to usurp an occupied cell was the only observed event which led to the disappearance of brood. Although the act of brood killing was only witnessed on 3 occasions, 60% of offspring lost were associated with usurpation events (in the remaining 40% of cases the mother lost her young but not her cell). Moreover, in Nest B, brood loss was common early in the observation period: the ratio of Sealed Cells (or successfully reared offspring to the prepupal stage) to Brood Loss (SC:BL) was 1.7:1. During this period, the average number of adult females for every open cell (♀♀:OC) was 1.29. The SC:BL ratio increased dramatically (7:1) after females were sacrificed for hormone assays, reducing the ♀♀:OC ratio to 0.76. In Nest A, the total number of newly vacated cells was not carefully tracked from day to day, but intermittent night surveys consistently revealed a nest with several

more adults than cells. Taken together, these observations strongly suggest that brood killing by competitors is the major cause of offspring disappearance.

Mothers of very young brood spent much of their time ensconced in their cell, which protected their young from usurpation attempts. Nonetheless, some females on occasion left their vulnerable young unguarded, and foraged for plant material which they applied to peripheral areas of the nest, usually the apex (Figure 1). This “team task” is usually carried out by females already foraging for plant material (e.g., building, closing or lining the inside of a cell) and may help with camouflage, nest reinforcement and/or as a buffer against parasitoids.

Brood development

The mean duration of development from egg to cell closure was 11.7 days (ranging from 9-15 days; n = 24) for Nest A and only 9.5 days (ranging from 7-11 days; n = 35) for Nest B. The average temperature for Nest A was 27.9ºC (range of 25.3-30.2ºC) with an average humidity of 80.6%. The mean temperature for Nest B was 26.7ºC (range of 24.7-29.0ºC) with an average humidity of 72.5%. Measures of rainfall were virtually identical. All climate data was collected from the Aeroporto de Aracaju, ~9 km from UFS (www.TuTiempo.net). Both nests were found on the same building, but on opposite sides. Nest A, which faced north, likely received less sunlight because of shadows casted by nearby trees whereas Nest B faced south toward an open field.

Brood parasitism or intranest prey theft

Consistent with earlier descriptions (West-Eberhard, 1987a, 2005), intranest prey theft was very common in *Z. miniatus* and the stealing of provisions typically occurred in one direction: toward cells containing older (i.e., larger) or same aged larvae. In Nest A, 31 unambiguous cases of theft (of many overall incidences) were recorded. Of these, there were 14 (45%) cases where prey was stolen from a larva at least 2 days younger than recipient larva (i.e., offspring of thief),

15 (48%) cases where the larvae were similarly aged (±1 day) and only 2 cases (6%) where the recipient larva was at least 2 days younger. Prey robbing was performed by females with both small and large larvae. Finally, I also observed three instances where prey was stolen directly from the mouths of nestmates returning from a successful hunt.

Adoption

As has been reported previously for *Zethus miniatus* (West-Eberhard, 1987a), I observed adult females caring for larvae which were not their own. In one of two cases, a mother who had recently been usurped adopted a mid-sized larva whose mother had disappeared. She reared her for 6 days and closed the cell. I also observed a recently eclosed female care for a *non-orphaned* larva. The mother and young 'helper' female often jostled with one another to access the larva within the cell, and on several occasions the mother displayed sting threats toward the helper. Even so, the helper foraged and stole provisions from others on the nest to feed the larva. After the cell was closed, the young female and mother parted ways and procured new cells, laid eggs and were never seen to interact again.

Ovarian growth

Mothers who lost young offspring were able to lay another egg within 2 days if they obtained a new cell (Figure 3A). Consistent with this observation, females caring for young larvae (i.e., provisioners; N=6) had one large vitellogenic oocyte (1.9 ± 0.42 mm, N=6) or 66% length of a mature egg (LME)) and a second developing oocyte adjoined to nurse cells with a constricted trophic stalk (0.9 ± 0.26 mm or 30% LME) (Figure 3B1) (N=6). Females who had recently sealed a cell and began inspecting other cells carried a fully or nearly full grown egg (2.5 ± 0.32 mm or 86% LME) and, on average, a larger second oocyte than provisioners (1.3 ± 073 mm or 51% LME) (Figure 3B2) (N=7). Both groups of females had an additional small, clearly pre-vitellogenic oocyte. Thus, the ovaries of *Z. miniatus* appear to be continuously active with oocytes in many stages of development rather than producing one egg at a time.

Maturing females (i.e., mated but prior to first oviposition) had a range of ovary sizes. Eclosion was rarely observed during the collection period, and so exact ages were not determined. The putatively youngest wasps, those who had not yet engaged in maternal behaviors, had primary oocytes ranging from 1.1-1.97 mm (or 38-69% LME) and secondary oocytes measured at 0.44-0.75 mm (or 16-26% LME). Young, pre-egg lay females engaged in cell acquisition behaviors had larger primary oocytes ranging from 2.1-2.93 mm (or 74-100% LME) and secondary oocytes spanning 0.5-1.36 mm (or 18-48% LME). None of the ovaries of these females had 'yellow bodies' (yb in Figure 3B), indicative of recent oviposition (Tyndale-Biscoe, 1984) or obvious irregularities such as opaque white regions suggestive of ovarian degeneration.

Juvenile Hormone

JH titers did not differ between 'provisioners of young brood' and females who had completed sealing a cell and began inspecting vacant or occupied cells on the nest ('cell seeking' females) (Figure 3C), nor did JH titers differ between pre-ovipositing and post-ovipositing females (Figure 4A). The great range of titers seen in the latter group (Figure 4A), which were assayed together, relate to the dates of collection and ♀♀:OC ratio (Figure 4B). All females collected on Nov. 11-12 ("early collection") had much higher JH titers than wasps collected from Nov. 18-23 ("later collection"), irrespective of female class. Meteorological data on campus report an aberrant rise in humidity, a drop in temperature and increased rainfall preceding the early collection of females while drier, more stable conditions persisted through the later collection. The lower JH titers were also correlated with the decline of the ♀♀:OC ratio (reported above). Thus, although JH titers can vary on a short time scale in *Z. miniatus*, they did not differ according to maturational state or maternal phase on the two nests studied. Instead, they vary according to climate, social competition or some other factor.

Discussion

Brood killing and its consequences: ovarian activity and JH titers do not correlate with the cyclical phases of maternal care in *Zethus miniatus*

The unexpectedly high rate of offspring mortality, due mainly if not entirely to usurpation attempts and intranest brood killing, might select for maintenance of active ovaries throughout the provisioning phase of maternal care. Accordingly, most of the mature females collected, regardless of their behavioral phase, possessed a nearly or fully mature egg accompanied by a second borderline vitellogenic oocyte. Obviously, the ovaries of an egg layer will be smaller after oviposition, but they do not appear to enter a quiescent phase or appear 'worker-like' as was envisioned for solitary progressive provisioners by the Ovarian Ground Plan hypothesis (West-Eberhard, 1987a, 1996). Continuously active ovaries may explain why JH titers do not associate with behavioral phases since JH functions as a gonadotropin across Insecta (Wyatt and Davey, 1996), including basal (Hatakeyama and Oishi, 1990) and primitively eusocial hymenopterans (Barth et al., 1975).

It is clear that newly eclosed females require at least a few days to a week to develop their first egg. Again, JH titers did not differ between females who had and had not yet oviposited (Figure 4A). Unfortunately, newly eclosed females were difficult to track during the observation period, and the possibility that JH titers rise after mating, coinciding with the onset of maternal behaviors [see *M. genalis* (Smith *et al*., 2012)], was not tested.

It is evident, however, that JH titers are not static: collections made a week apart from the same nest (and assayed together) showed a striking difference in JH titer, irrespective of maturational state (Figure 4B). This may have been caused by a shift in the climate, or as seems more likely, by a shift in the ratio of adult females to available cells. JH titers were elevated only when social competition for cells was high (i.e., when the ♀♀:OC was greater than 1). When cells

outnumbered females, resulting in less competition, JH titers were extremely low. Although preliminary, the pattern is reminiscent of primitively eusocial paper wasps where JH titers are upregulated in periods of social competition (e.g., in a queenless nest) (Tibbetts and Huang, 2010). Vertebrate endocrinologists will recognize the implication: JH in wasps may have functions analogous to testosterone (T) in vertebrates, particularly birds (Tibbetts and Huang, 2010). The empirically supported 'challenge hypothesis' predicts that T titers will increase in periods of male-male competition, providing a boost to an individual's fertility and mating success but at the expense of a compromised immune system and greater mortality (Wingfield et al., 1990). It is well established that JH or methoprene (a JH mimic) application induces aggression (Strambi, 1990) and ovarian maturation (Barth et al., 1975) yet reduces survival in queens and well-fed workers of *Polistes dominulus* (Tibbetts and Banan, 2010). In *M. genalis*, JH titers are higher in queens than in same aged solitary females (Smith *et al*., 2012). Whether or not social competition truly influences JH levels in *Z. miniatus* (and is widespread among group-living or even cluster-nesting wasps and bees) will require detailed observations of behavior in nests with low (♀♀<OC) and high (♀♀>OC) levels of usurpation.

Group life among and despite brood killers

Brood killing and usurpation is probably the most significant cost of communal life in *Z. miniatus.* Ironically, a putative major selective advantage of switching from limited to extended brood care is the increased protection of young offspring. As Evans (Evans, 1966) notes, "there is little question that there is far less mortality to eggs and small larvae in progressive provisioners than in mass provisioners". The advent of group life, then, seems to have reversed the reputed chief benefit reaped by solitary, progressive provisioning antecedents of *Z. miniatus*. Nonetheless, the fact that the majority of females remain on their natal nest instead of founding elsewhere (West-Eberhard, 1987a) suggests the benefits derived from social living, namely, cell re-use, prey exchange, group defense against natural enemies and assured fitness returns (e.g.,

occasional brood adoption) outweigh the accumulated costs of resource competition, cell usurpation and brood killing. A quantitative assessment of the cost and benefits of group life for *Z. miniatus* should be feasible since new nests are occasionally founded by one or more females close to (and derived from) large, established nests. It would be interesting to compare, for example, the duration of brood development, time spent guarding brood, provisioning patterns, and overall reproductive success in nests with varying number of females.

Does *Z. miniatus* truly represent an intermediate stage of evolution between solitary and eusocial wasps? Arguments for and against:

Z. miniatus exhibits many behavioral traits reminiscent of primitively eusocial wasps which, on the surface, justify their use in research as a proxy for an intermediate stage of evolution between solitary and eusocial wasps (West-Eberhard, 1987a, 2005). First, they are progressive provisioners, relatively rare among solitary vespid wasps but widespread in eusocial species (see West-Eberhard, 2005, Table 1). Second, orphaned larvae are occasionally adopted by females with putatively less reproductive potential (West-Eberhard, 1987a), and as observed here, maternal care can be stimulated before oviposition, a requisite step toward the realization of a worker phenotype (Linksvayer and Wade, 2005). Third, not all adult wasps are created equal (i.e., larval nutrition, genetics), and as West-Eberhard (West-Eberhard, 1978) documented, this can lead to rudimentary reproductive dominance with some females raising nearly twice as many offspring as others over a 2 month period. Fourth, as emphasized in this report, differential egg destruction and usurpation by aggressive females is a hallmark of eusocial queens (West-Eberhard, 1969). The act of egg guarding, too, may correspond to the post-oviposition vigil seen in Epiponini swarm founding wasp queens (e.g., *Metapolybia aztecoides* (West-Eberhard, 1977, 1981), which increases with increased competition. Fifth, the performance of ‘team tasks’ in the face of risk (e.g., nest maintenance and nest patrolling) are suggestive of worker tasks. Sixth, females will oviposit into partially constructed cells, a behavior

commonly observed in eusocial wasp queens. Finally, *Zethus* belongs to the subfamily Eumeninae which is sister group to all eusocial vespid wasps. [*Note*: A multigene phylogeny placed 'Zethinae' basal to the majority of eusocial vespids (Hines et al., 2007)]. However, a more complete and empirically superior study all but demolished this scenario (Pickett and Carpenter, 2010), restoring *Zethus* as an unambiguous member of Eumeninae].

Although *Z. miniatus* may be ancestral-*like* and is a member of a subfamily closely related to eusocial subfamilies, it is important to emphasize that not a single eumenid wasp (of ~3000 species) has attained eusociality (Pickett and Carpenter, 2010). Wcislo and Tierney (Wcislo and Tierney, 2009) note "a striking fact about the phyletic position of communal behavior in aculeate Hymenoptera is that it typically occurs in clades in which there are no examples of caste-based societies" and the Eumeninae are no exception. It is also noteworthy that *Z. miniatus* females do not fight or compete in a way that leads to consistent winners and losers necessary for a division of labor to emerge (cf. all primitively eusocial paper wasps). Indeed, despite their readiness to usurp and kill the young brood of a neighbor, they resist stealing provisions from cells containing larvae larger than their own. This adaptation of restraint, presumably the result of mutualism or genetic relatedness, is perfectly suited for communal life, serving as a sort of insurance against the loss of a significant investment by a nestmate. Thus, we must keep in mind the possibility that a caste-based society simply cannot be easily derived from the specialized communal arrangement of *Z. miniatus*.

Future explorations of the Ovarian Ground Plan

The Ovarian Ground Plan cannot be rejected based on a study of a single sub-eusocial species, especially a species in which brood killing is common. The best strategy may be to study large solitary progressive provisioning wasps since they probably suffer much lower rates of young offspring mortality (Evans, 1966). Also, large-bodied species can spend up to a month provisioning for a single larva (Longair, 2004; Roubaud, 1910), perhaps allowing for a more

pronounced cycle of ovary growth and higher volume haemolymph samples. Indeed, the original inspiration for the Ovarian Ground Plan came from Iwata's survey (Iwata, 1955) of ovarian anatomy across Hymenoptera (West-Eberhard, 1987a) which was highlighted by Evans (1966). In a "crude" analysis of Iwata's data, O'Neill (O'Neill, 2001) reveals a reduction in egg and ovariole number and a concomitant enlargement of egg size as solitary wasps shift from parasitic to nest-provisioning instincts, with the "extreme condition" represented by solitary wasps engaged in extended brood care. The individual histories of these wasps, whose ovarian state was evaluated based on one (*B. japonicas*) or two dissected specimens, were not known. In addition, there are other primitively social wasps with behavioral repertoires very similar to *Z. miniatus* but observations of brood killing have not been reported (Evans, 1973; West-Eberhard, 1987b; West-Eberhard, 2005). Even if brood killing is a regular occurrence on the nests in other group-living wasps, this would be a great opportunity to independently verify whether JH is up-regulated in the context of social competition (e.g., for open cells).

An alternative hypothesis for the origin of castes, which relies almost entirely on data from temperate species of *Polistes*, supposes that the emergence of an adult diapause state in solitary wasps set the stage for nascent castes (Hunt, 2006; Hunt and Amdam, 2005). Yet the Diapause Ground Plan (Hunt 2006, 2007) is not a feasible scenario since it assumes, contrary to the evidence (Lecht, 1964; Pickett and Carpenter, 2010), a temperate zone origin of queens and workers. Clearly, the most primitively eusocial vespid wasps are the socially varied hover wasps (Stenogastrinae) of Southeast Asia, convincingly demonstrated to be basal to other eusocial vespids (Pickett and Carpenter, 2010). A study of their endocrinology should be a top priority (although most species are too small for more accurate techniques such as RIA).

The mechanism of caste origin in vespid wasps remains unverified, although extensive comparative studies by West-Eberhard (1978; 1987a; 1996) makes the Ovarian Ground Plan hypothesis viable. Indeed, as predicted (West-Eberhard, 1996), empirical research on

primitively eusocial wasps supports the notion that JH has condition-dependent effects (Giray et al., 2005; Shorter and Tibbetts, 2009; Tibbetts et al., 2011). Although this report has emphasized the importance of progressive provisioning as an important preadaptation for incipient castes, it certainly is not the only route, since the mass provisioning halcitid bees have evolved castes 2 or 3 times (Gibbs et al., 2012). If there is a fundamental hymenopteran ground plan which is conducive for caste creation, we would expect parallel endocrine profiles to emerge in distinct eusocial lineages regardless of the route (semi-social vs. matrifilial) taken. Established as well as recent advances in applicable techniques (e.g., RNAseq) make the question of multifarious caste origins an area ripe for modern research. For example, how were JH, other behavioral modifiers and gene networks co-opted for social life in small carpenter bees, apoid wasps and the facultatively eusocial hover wasps? In all these groups, do we see a surge in JH in the face of social competition? Will we uncover evidence for JH function in subordinate, under-nourished females caring for the young of others? Such studies, when focused on the ancestral solitary ground plan, will help assess the importance of phenotypic variation (vs. genetic change) in the macroevolutionary shift toward caste-based societies.

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Figures

Figure 1. Nest of *Zethus miniatus* (Nest A in text). The nest was founded on a wire fixed to an eave of a building. The material of the nest is made from masticated plant material, the green parts being the freshest additions to the nest (* indicate new cells). Only some cells are visible in this photograph since cells can face in opposite directions. + indicates a cooperatively built structure which does not relate to cell construction (most nests featured these amorphous structures, always manifesting at the apex of the nest). Most wasps can be seen ensconced in their cells; paint marks are evident on the two females at rest (arrow heads).

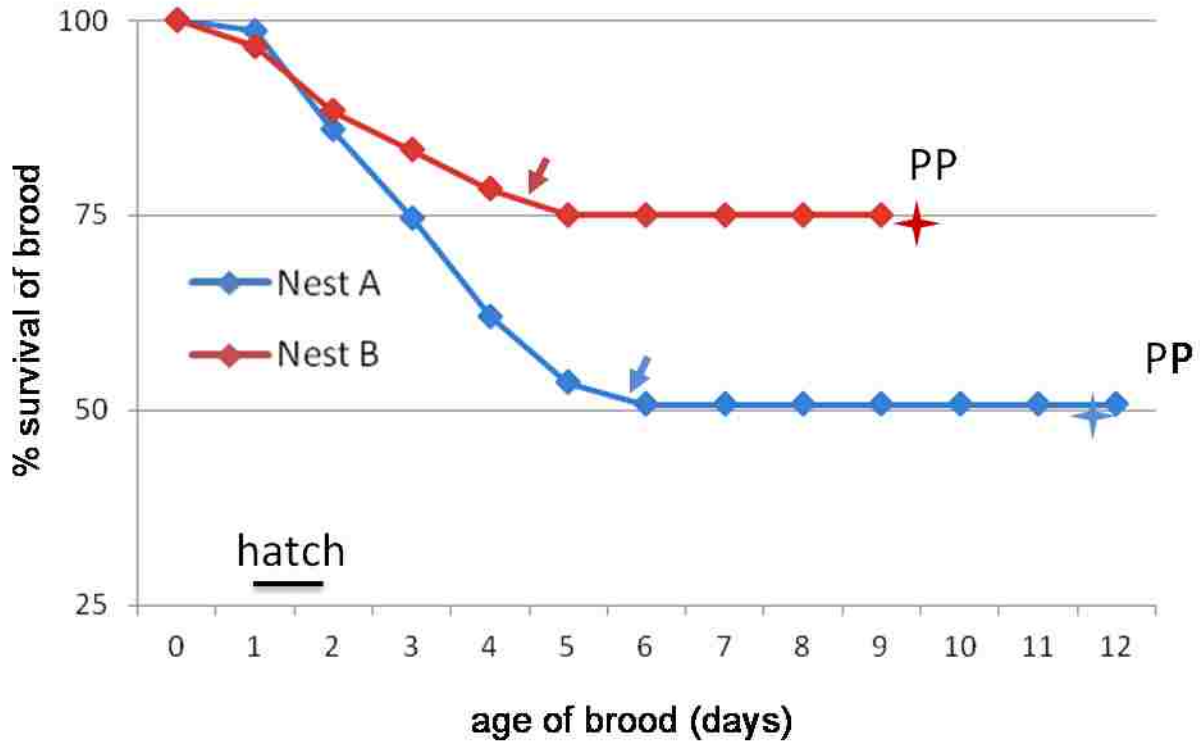

Figure 2. Brood disappearance data. Per cent survival was calculated separately for each nest by dividing the number cell closures by the number of brood disappearances. The survival lines end on the median number days required to rear an egg to a prepupa (PP); stars represent the mean for each nest. Arrows indicate halfway point of egg and larval development. Hatching occurs 1 or 2 days after an egg was first recorded.

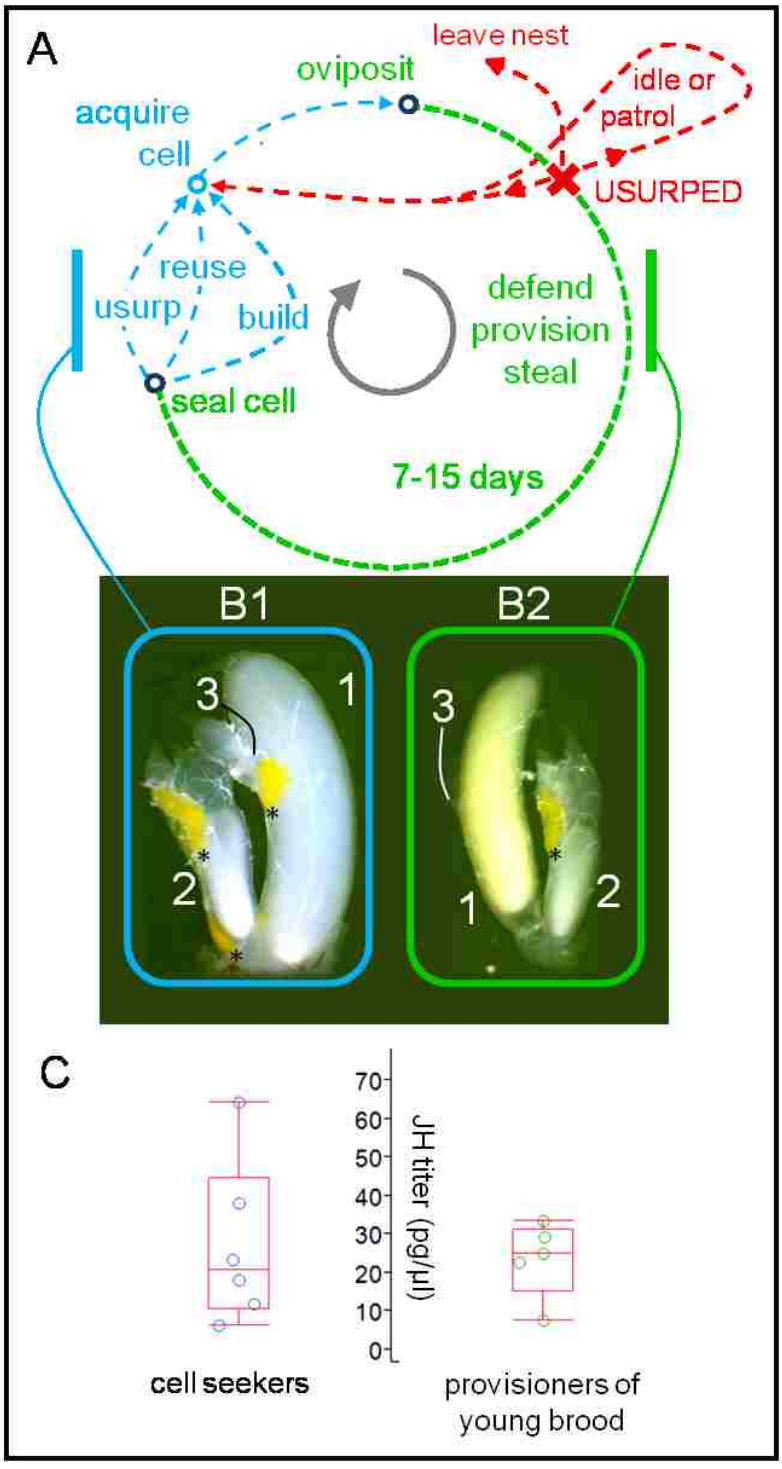

Figure 3. Summary of behavioral patterns, ovarian growth and JH titers in mature females of *Z. miniatus*. (A)The dark blue lines and text correspond to brood care. Red lines and text indicate behavioral responses of victims to cell usurpation events (option of adoption not shown). Light blue lines and text show alternative strategies for cell acquisition. Bars indicate phases where females were sacrificed for (B1-B2) ovarian (numbers indicate oocytes; * indicate yellow bodies) and (C) JH titer measurement.

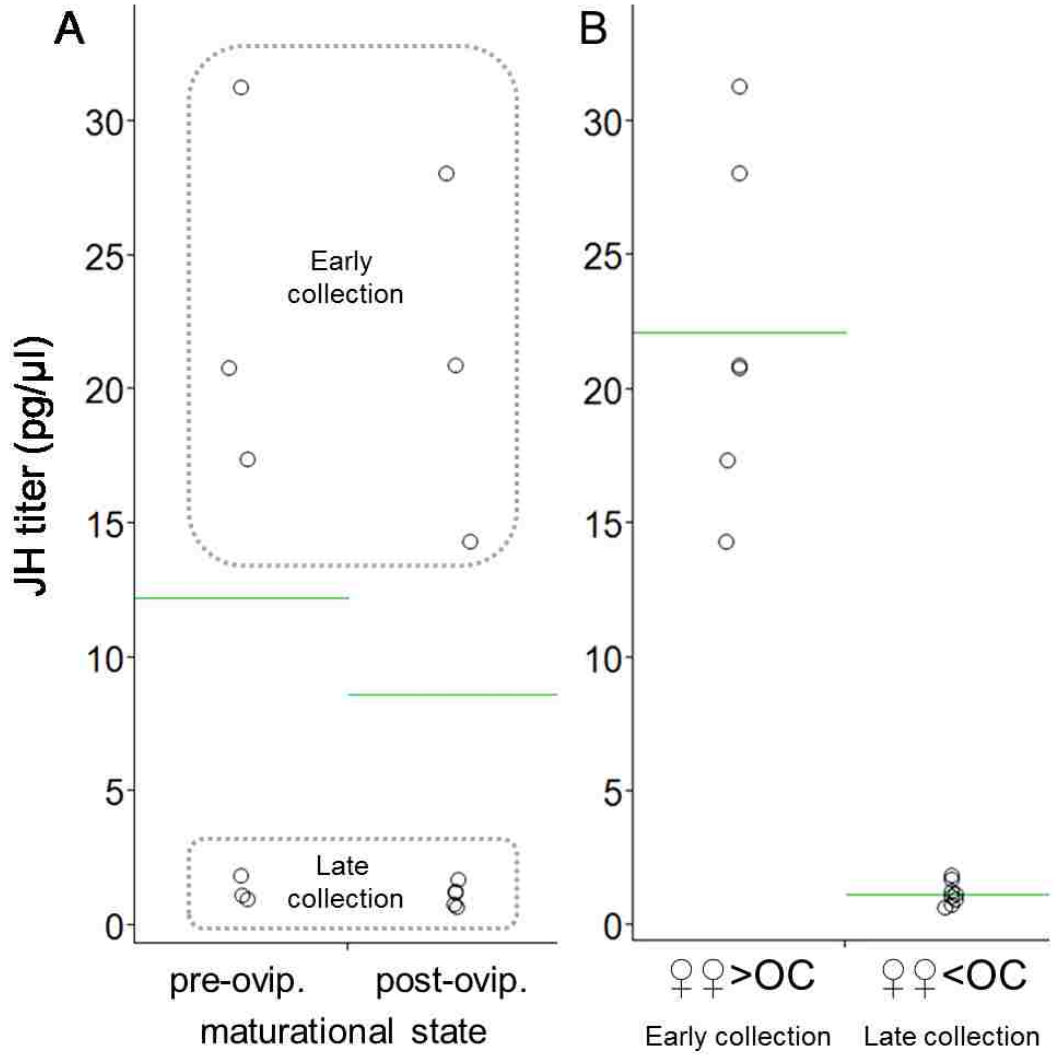

Figure 4. (A) JH titers of females before ("pre-ovip.") and after ("post-ovip.") oviposition from Nest B. Females were collected either from Nov 12-13 ('Early collection') or Nov 18-23 ('Late collection'). (B) Same samples from A but separated by collection period ('Early' vs. 'Late') which also corresponds to whether females outnumbered (♀♀>OC) or were outnumbered (♀♀<OC) by available cells, a possible indicator of competition (see text).

Chapter 3:
The endocrinology of *Polybia micans*

Table of Contents

Introduction

Advanced or temperate societies of eusocial insects are characterized by the production of at least two distinct phenotypes: the queen, the worker, and a third one, the soldier, in ants and termites(Wilson, 1971). In most species, caste phenotypes are realized or at least biased in the pre-imaginal stage, where differential diet, pheromonal or mechanical signals trigger endocrine responses which lead to differential development, producing alternative phenotypes (Hartfelder

and Emlen, 2011; Suryanarayanan et al., 2011; Wilson, 1971). As with all polyphenisms in insects, juvenile hormone (JH) and the ecdysteroids conspire to orchestrate distinct ontogenetic trajectories on top of their well-conserved role in regulating the episodic, molt-based growth in immatures (Hartfelder and Emlen, 2011). This is typically accomplished by particular hormone-sensitive periods which serve as bifurcation points in development (Hartfelder and Emlen, 2011; Nijhout, 1994).

Following eclosion in most insects, JH assumes a new function: reproduction. Typically, JH stimulates the fat body to synthesize vitellogenin, a yolk protein, and/or stimulates the follicle cells of the germarium to separate, allowing for the uptake of vitellogenin into the oocyte (Nijhout, 1994; Wyatt and Davey, 1996). A gonadotropic function for JH is conserved in primitively eusocial species, like bumble bees (*Bombus*) and paper wasps (*Polistes* and *Ropalidia*) (Agrahari and Gadagkar, 2003; Bloch et al., 2002). In the case of *Polistes*, JH and ecdysteroids regulate dominance behavior among competing foundresses (Roseler et al., 1984; Tibbetts et al., 2011) and JH has also been implicated to control fertility signaling through cuticular hydrocarbons (CHCs) (Izzo et al., 2010). However, in more highly advanced societies, such as honeybees, stingless bees and secondarily queenless ants, JH has either no function at all or actually reduce fertility in reproductives (Brent et al., 2006; Hartfelder et al., 2002; Hartfelder and Engels, 1998; Penick et al., 2011). Instead, JH titers are higher in foraging workers. As had been convincingly demonstrated in honeybees, JH modulates the onset of foraging, an age-related behavioral and physiological transition (Huang et al., 1991; Robinson and Vargo, 1997). A similar function is operational in ants (Brent et al., 2006; Dolezal et al., 2012; Penick et al., 2011), *Polybia occidentalis* (an epiponine swarm founding wasp) (O'Donnell and Jeanne, 1993) and *Polistes* wasps (Giray et al., 2005; Shorter and Tibbetts, 2009). In *Polistes* it has been demonstrated that JH can be gonadotropic in one phenotype while functioning as a behavioral ‘pace-maker’ in another (Giray et al., 2005). Response to JH mimic

treatments in *Polistes* reveals that an individual's physiological condition is the key: well-fed females respond with ovarian growth and dominance (Roseler et al., 1984; Tibbetts and Izzo, 2009) while undernourished females respond by foraging precociously (Shorter and Tibbetts, 2009).

Many of the Epiponini swarm founding wasps of the Neotropics defy typical classification of primitive vs. highly eusocial in that some species do not have morphologically or even physiologically distinguished castes yet are obligately eusocial. They have few to many queens which engage in ritualized signaling, the colonies reproduce by swarming and colony membership often reaches well into the thousands (Jeanne, 1991; West-Eberhard, 1978a). Newly eclosed individuals have the capacity to become a queen or worker, and their physiological development depends on the social context (West-Eberhard, 1978b). The major benefit for determining caste in the adult stage appears to be flexibility. Raids by army ants in the Neotropics have been postulated to represent a major selective pressure for maintaining prolonged caste flexibility in swarm founding wasps: if a nest is overrun by ants, the colony can quickly disband and establish a nest elsewhere, and if the queens are lost, new queens are selected from a standing pool of adults (Jeanne, 1991; West-Eberhard, 1977). Thus, analogous to the hemimetablous termites, epiponine colonies demonstrate remarkable resilience to foreign assaults by maintaining totipotency as active members of the colony. But unlike the termites, caste differentiation and mechanisms regulating caste physiology and behavior are all processes which take place in the imaginal stage.

Polybia represents the most derived and speciose genus of wasps within the Epiponini (Pickett and Carpenter, 2010). In general, *Polybia* queens are perceptibly or only statistically larger than workers (Noll et al., 2004; Noll and Zucchi, 2000). Such determinations are often problematic for Epiponini due to the way in which their colony demographics fluctuate (Noll and Zucchi, 2000). When a colony swarms, the colony has many queens, which results in laying as many eggs as

possible in the newly constructed nest. In the “astelocyttarus” clade of wasps, which have a nest architecture ideal for observation (*Metapolybia*, *Synoeca*, *Asteloeca*) (see Chapter 4), workers challenge queens by performing the ‘queen dance’, a spasmodic act of inhibited aggression which challenges the dominance of the passing queen. So long as the queen appeases the worker, often by responding with a lateral sting-like posture which is thought represent a ritualized dominance display (‘abdomen bending’), the queen is not attacked. However, if the queen surrenders nutritive saliva (via trophallaxis) or crouches in response to the engaged worker, both of which are perceived as submissive acts, the queen will be attacked, subjugated and at the very least, relegated to worker status. Through interactions like these and other means of queen attrition, the queen number eventually dwindles down to very few or even one queen, and the nest becomes monarchic (West-Eberhard, 1977, 1978b). Eventually, if reproductive conflicts occur (e.g., young females are able to mature eggs in the presence of the lone queen), the colony fissions and the young reproductives, accompanied by a portion of workers, swarm to a new location (Jeanne, 1991). This colony cycle, called ‘cyclical oligarchy’, leads to fluctuating degrees of dimorphism between the castes as larger queens appear to have a slight advantage in fecundity over others (West-Eberhard, 1978b). Because workers do not face such selective social pressures, a difference is detected based on the subtraction of smaller egg layers from the cohort of queens. Thus, whether a species has been designated as dimorphic or not often depends on when in the cycle the colony was sacrificed for morphometric analysis (Noll and Zucchi, 2002). In terms of development, however, there do not appear to be any ontogenetic switch points, although a better fed larva would appear to have an advantage for sustaining high fecundity or dominance if she is afforded an opportunity to become a queen.

Nothing is known about the biology of *Polybia micans* aside from a brief note claiming they have monomorphic castes (Richards, 1978), indicating that caste determination occurs in the adult stage. Indeed, the mode of queen replacement reported herein demonstrates this. The main

objective of this work was to describe the endocrinology of a highly eusocial species (from a much neglected taxon) to test how hormone functions change as lineages evolve more sophisticated and complex societies. Given the roles of JH and ecdysteroids in dominance, and JH in reproduction and fertility (CHC) signaling in competing foundresses and prospective reproductives of *Polistes* (Izzo et al., 2010; Roseler et al., 1985; Roseler et al., 1984; Tibbetts and Huang, 2010; Tibbetts et al., 2011), should we expect the same functions to be preserved in the more highly derived polygynous *P. micans*, where reproductive competition is constant? Or might we expect a decreasing dependence on JH to function as a gonadotropin with increasing societal evolution, giving way to a more specialized worker-specific role for JH, as was suggested by methoprene treatments for *P. occidentalis* (O'Donnell and Jeanne, 1993)? The answer is somewhere in between, and often outside the predictions of the above hypotheses. In addition to characterizing the endocrinology of *P. micans*, this is the first report of ritualized displays and queen replacement mechanisms in a non-astelocyttarus clade.

Methods

Wasps

All behavioral and physiological data were collected from *Polybia micans* [Hymenoptera: Vespidae: Epiponini].

Field studies

Field work was carried out on the campus of Universidade de Federal Sergipe (UFS), São Cristóvão, Sergipe, Brazil. Nests were found affixed to a variety of substrates, including tree trunks, tree branches and man-made structures. Several attempts were made to transfer nests to the more convenient locations and height, but this usually resulted in swarming or a much reduced level of observable activity. Colony 6 is the only nest included here which did not show

a marked difference in activity after being transferred, and two weeks were allowed to pass before observation began. All other colonies reported here were studied *in situ*.

The paper nest architecture of *P. micans* consists of horizontal convex combs which are encapsulated by an envelope, with an opening positioned at the bottom (Figure 1). Observation into the interior of a nest is therefore problematic, and I found cutting into completely enveloped nests frequently resulted in swarming. I thus sought out colonies in a period of nest growth since it provides a natural, temporary window to observe activity inside the nest. When the colony decides to expand, some workers begin building cells on the bottom outside of the envelope while others initiate construction of a new envelope which will eventually surround the new cells, creating the next comb. I found that I could perpetuate this nest growth phase by daily removing recently built sections of the envelope, facilitating visual access into the outermost comb for a few days or a couple weeks, if needed. This technique rarely induced swarming (but see C7 in Results) since most of the observed worker activity was already dedicated to building. All non-founding colonies were in a phase of growth when observations began.

The appearance of empty cells is attractive to queens, and the technique described above enabled the collection of queens without destroying the colony. The emergence of queens from nest interior was often brief, there presence betrayed by the attention received from workers. Using bayonet forceps, females to be sacrificed were gripped by the legs or wings, and queens were removed as soon as their status was clear. It was impossible to know precisely how many queens were present in a given colony. In cases where there were many queens, and the objective was to remove them all, a full week of continuous observation was required.

Wasps were color marked with oil-based Sharpie pens according to the task performed (building, pulp foraging, meat foraging and liquid foraging). To avoid possible circadian endocrine changes (Zera, 2007), the vast majority of wasps were collected for processing from

13:00-17:00h. Wasps were placed in clean glass vials and buried in ice (0 ° C) within 15 seconds of removal. Collection events for individual wasps always included multiple types of females, with the relevant pair determined by the objective of the assay (e.g., queens vs. workers).

Some assays required the removal of entire nests. One objective for collecting colonies was to access young females from inside the nest which may not emerge very often from the interior. To facilitate the odds of finding these reclusive females for hormone measurements within an allotted time (see below), the nest was agitated for 5-10 seconds before collection in order to encourage the emergence and elimination of worker guards which were not targeted for study. The nest was then captured with a loose butterfly net, placed in a plastic bag and immediately buried in ice, where it was gently crumpled to allow the cool temperatures to penetrate all areas of the nest.

Laboratory procedures and protocols

Procedure for collecting haemolymph, cuticular hydrocarbons and ovary measurements:

Wasps were transferred on ice to the lab for processing. After 20-120 minutes of 0ºC, the anesthetized wasp was removed and immobilized against a substrate with intersecting insect pins under a stereomicroscope. Since cold anesthesia for a period exceeding 2 hours is known to significantly affect juvenile hormone titers in honeybees (Lin et al., 2004), all wasps were bled within this time frame unless otherwise noted. Using a 5 µl graduated microcapillary (Drummond Scientific Company) pulled to a point over a flame, 1 to 5 µl of haemolymph was withdrawn from between the anterior-most segments of the gaster. Samples destined for JH measurement by radioimmunoassay (RIA) were transferred to 500 µl of acetonitrile inside a 2 ml screw-top glass vial capped with a Teflon-lined rubber septum. Samples destined for ecdysteroid measurement were preserved in 500 µl of methanol. Subsequent to bleeding, cuticular hydrocarbons (CHCs)

were extracted from females by placing them in 2 ml of hexane for 2-2.2 minutes. The ovaries were carefully removed in cold E&B Ringer's solution (7.5g NaCl and 0.35g KCl / 1L distilled water (Ephrussi and Beadle, 1936)), photographed with a Leica EZ4D Microscope Camera and were measured using Leica Application Suite software (Leica Microsystems). Ovaries were then placed in 500 µl of methanol for ecdysteroid measurement. All hormone and CHC samples were kept at -20ºC until transport to the Universidade de São Paulo, Ribeirão Preto, where they were processed.

Juvenile hormone sample preparation (extraction)

JH was extracted from the haemolymph following a liquid-phase separation protocol developed for honeybees (Huang et al., 1994). The acetonitrile extract was combined with 1 ml 0.9% NaCl and 1 ml hexane in a 5 ml glass tube. After a thorough vortexing, the phases were allowed to separate on ice for 10 minutes, followed by centrifugation at 700 *g*. The supernatant hexane phase, containing the JH, was transferred to a new tube and the hexane extraction was repeated twice. The pooled hexane phases were dried by vacuum centrifugation and the extraction residues were redissolved in 50 µl toluene (containing 0.5% propanediol) and transferred to RIA glass vials. Just prior to the RIA, the solvent was removed by vacuum centrifugation.

Juvenile hormone titer analysis by radioimmunoassay (RIA)

The RIA was performed according to the protocol devised by Goodman *et al*. (Goodman et al., 1990). In preparation, the JH-specific antiserum was diluted 1:1250 in phosphate buffer containing 0.1% bovine serum albumin and 0.1% rabbit immunoglobulin G. As a tracer, we used [10-^{3}H(N)]-JH III (spec. activity 19.4 Ci/nmol, Perkin Elmer Life Sciences, Waltham, MA, USA), diluted in 0.1 M phosphate buffer (pH 7.2-7.4) (made from 0.2 M mono- and dibasic potassium phosphate stock solutions and 0.02% sodium azide) to 6000-6500 cpm/50 ml. Synthetic JH III (Fluka, Munich, Germany), the lone species of JH known in Hymenoptera (Goodman and

Cusson, 2011), was used as a non-radioactive ligand. Two standard curve replicates were set up to cover a range of 25 pg – 5 ng.

After an overnight incubation at 4ºC, saturated ammonium sulfate was added (50% final concentration) to precipitate the antibody-bound JH-III. Standard curve values were log/logit transformed, and a linear regression was produced to determine JH-III equivalents (pg/ml hemolymph) (i.e., titers) for each sample.

Ecdysteroid sample preparation

Haemolymph samples were cold centrifuged (4^{o}C) for 10 minutes to pellet the protein precipitate and impurities. The supernatant was directly transferred the RIA glass vials and dried by vacuum centrifugation.

To measure the ecdysteroid content of queen ovaries, which are particularly rich in lipids (which interfere with ligand-binding and the precipitation of the antigen-antibody complex), an additional purification step is needed. After decanting an aliquot or the entire supernatant of the ecdysteroid sample, the methanol was evaporated by vacuum centrifugation and resuspended in 1 ml 30% methanol. The sample was then slowly loaded into SepPak-C18 cartridge (Waters, WAT051910) with a 1 ml disposable syringe. After twice passing 1 ml 30% methanol through the cartridge, the eluate (containing polar ecdysteroid conjugates) was discarded. The cartridge, containing the free ecdysteroids, was eluted by two consecutive rinses with 60% methanol, and the pooled eluates were then dried by vacuum centrifugation and redisolved in 100% methanol (Geva et al., 2005).

Ecdysteroid analysis by radioimmunoassay (RIA)

Ecdysteroids were quantified by radioimmunoassay, as previously described (Feldlaufer and Hartfelder, 1987), using an antiserum prepared against a hemisuccinate derivative of ecdysone (Bollenbacher et al., 1983; Warren and Gilbert, 1986). In preparation for the RIA, the 20E-

specific antiserum was diluted 1:1300 in 0.1 M phosphate buffer (pH 7.2-7.4) (made from 0.2 M mono- and dibasic potassium phosphate stock solutions and 0.02% sodium azide) containing 0.1% bovine serum albumin and 0.1% rabbit immunoglobulin G. As a tracer, we used [23,24-3H(N)]ecdysone (Perkin Elmer) (NEN, spec. act. 102 Ci/mmol), diluted in 0.1M phosphate buffer (same as for JH-RIA above) to 5000-6000 cpm/100 ml. Standard curves were established using 20-hydroxyecdysone (20E; Sigma) as a nonradioactive ligand. Accordingly, results are expressed as 20E equivalents (pg). Two standard curve replicates were set up to cover a range of 25 pg – 2 ng.

After an overnight incubation at 4ºC, saturated ammonium sulfate was added (50% final concentration) to precipitate the antibody-bound 20E. Standard curve values were log/logit transformed, and a linear regression was produced to determine 20E equivalents for ovarian content or titers for each sample.

Cuticular Hydrocarbon Analysis

The epicuticular surface hydrocarbons were extracted in 2 ml hexane for 2-2.2 minutes. After the solvent was evaporated under a fume-hood, the apolar extract was suspended in 50 µl of hexane. One µl of this was injected into a combined gas chromatography-mass spectrometer (Shimadzu, model QP2010). Separation was achieved on a DB-5MS column of 30 m, with a helium gas carrier at 1.0 ml min^{-1}. For most samples, the oven temperature was initially set to 150º C, and ramped up 3º C min^{-1} until it reached 280ºC and held for 20 minutes. Analyses were performed in the splitless mode. The mass spectra were obtained by 70 eV ionization. The chromatographs were analyzed with GCMS solutions (Shimadzu).

Statistical analyses

Hormone data

I used only non-parametric tests in the statistical analysis of hormone titers and ecdysteroid content of the ovaries because these variables were not normally distributed (Shapiro-Wilk W test). The Wilcoxon (or Mann-Whitney U test) was used for comparing groups using JMP 10.

Cuticular hydrocarbon data

Only compounds which were shared among or between colonies were used in the discriminant analyses, and the relative concentrations of the compounds were readjusted to 100%. Principal Component Analysis (PCA) was not used to eliminate variables from the Discriminant Analysis because the number of shared compounds from pooled colonies was low (from 13-18). Following the DA, a stepwise discriminant function analysis was used to observe if combinations of variables could be useful in the predicting group. Wilks' λ values were used to verify the individual contribution of each variable to the model.

To avoid errors in the compositional sample data, the area under each peak was transformed according to the following formula: $Z = \ln[Ap/g(Ap)]$, where Ap is the area under the peak, $g(Ap)$ is the geometric mean for each individual compound group and Z is the transformed peak area (Aitchison, 1986). We compared proportions of compounds for each wasp group using the *t*-test. The statistical analyses were performed using the software Statistica 10.0 (Statsoft, Inc.).

Determination of ovarian status

The stage of ovarian development among non-queens was scored based on a pictorial index presented in Figure 2. Stage A ovaries are filamentous, bearing no discernible oocytes. The ovarioles of stage B ovaries are irregular and bulbous, comprised of very small (and likely degenerate) oocytes. Stage C ovaries contain discernible oocytes but are not as pronounced as Stage D ovaries which feature spherical oocytes adjoined to spherical trophocyte chambers of

comparable length. Stage E ovaries bear oocytes which take on an oval shape and are perceptibly longer than the adjoining trophocyte chamber. Such elongated oocytes are likely taking in vitellogenin by endocytosis. Vitellogenesis is further evidenced by an opaqueness of the oocytes at this stage. Further development will result in the complete absorption of cytoplasm from the trophocyte chamber, marking the onset of Stage F ovaries. Stage G ovaries have produced or are producing 1-3 chorionated oocytes (i.e., eggs). Stage H ovaries are queen-like, bearing many eggs.

Queen ovaries were quantified by counting the number of oocytes > 75% the length of a normal egg.

Determination of relative age

Relative age was estimated by scoring the degree of apodeme darkening on the 5th sternite (West-Eberhard, 1969). Pictorial representations of this feature and the scores assigned are shown in the top of Figure 3.

Results

Life History

Nests were found in a variety of states (Table 1). Each non-founding colony (Colonies 1-8) was in a period of nest expansion which was perpetuated by daily removal of newly constructed pieces of the envelope, providing a window into outermost comb of the nest (Figure 1). C7 swarmed three times over the course of a month. They were initially observed in an established, four comb nest, but the colony swarmed to an unknown location for three weeks before returning to their previous location, whereupon they established a new nest of 3 combs. Perhaps due to observer manipulation of the nest envelope, they swarmed again, founding a

new colony on an adjacent branch. Colonies 9 and 10 were discovered as they were founding nests.

Colony	Nest state	Queen number	Number of oocytes > 75%	Worker number	Comb layers	Males?	Month
1	post-emergence	1	lost data	~300	3	yes	March
2	post-emergence	>18	15.9 (14-20)	~500	3	no	May
3	pre-emergence	2	20 & 22	22	2	no	June
4	post-emergence	1	35	~350	3	yes	June
5	pre-emergence	19	23.6 (16-31)	~400	4	no	February
6	post-emergence	>27	4.7 (3-7)	~300	3	no	April
7	multiple	>11	14.7 (7 & 12-22)	~200	4→3→1	no	April
8	post-emergence	2	32 & 34	~300	4	no	May
9	founding	>>4	18.5 (16-20)	~200	1	no	September
10	founding	>>5	7.2 (5-9)	~300	1	no	November

Table 1. Basic information for colonies included in this report. Nest state indicates whether a nest is in a founding or established phase, and if the latter, whether the nest had begun to produce adults. Exact queen number was known for only some colonies, and worker number was estimated. Average number of oocytes is given along with the range within a given colony. Comb layers indicate the size of the colony. Colony 7 was found to occupy three different nests. Originally observed as a 4 comb nest, they swarmed frequently, creating at least two more short-lived nests of 3 and 1 combs, respectively. All colonies except Colony 3 were used for hormone assays, and Colonies 2-8 were used for cuticular hydrocarbon analyses.

Queen number ranged from 1 to over 28. There were no obvious differences in morphology between queens and workers aside from slightly swollen gasters in very fecund queens (body and wing measurements were not performed). Ovary size (i.e., oocytes >75% the length of a fully grown egg) was inversely related to queen number in established colonies where the exact (Colonies 1, 3-5, and 8) or approximate (Colonies 2 and 6) number of queens was known (ANOVA, $P=0.0375$). A significant relationship held even if an additional 5 queens were added to either or both Colony 2 and Colony 6 where queen number was underestimated ($P<0.05$).

Analysis of the cuticular character on the 5th abdominal sternite (Fig. 3) revealed that queens from a given nest were similarly aged.

A range of ovary sizes among queen cohorts was notable in some nests, such as Colony 5 (Table 1) but rarely extreme. In the established first nest of Colony 7, one queen was found lacking eggs entirely. The proximal areas (i.e., oviduct-side) of her ovarioles were devoid of oocytes and instead were full of yellow bodies, indicative of a long history of ovipositions (Tyndale-Biscoe, 1984).

Behavior

In accord with studies on astelocyttarus Epiponini wasps (Nascimento et al., 2004; West-Eberhard, 1977, 1978b), workers performed the 'queen-dance', which the queen would either ignore or perform the abdomen-bending display. Although the propensity for bending was not recorded in detail, single queens were by far the most active (i.e., aggressive) benders. The eggless, defeated queen from Colony 7 was found on the very periphery of the outmost comb, a rare resting place for a queen. Passing workers would perform the queen-dance toward her and occasionally bite, although the confrontations were never observed to escalate to outright attacks.

Except in colonies with many queens (e.g. Colonies 2 and 6), relatively young females showed some ovarian maturation (B-E type ovaries) whereas older females tended to have filamentous ovaries. In colonies where all queens were eliminated and the nest was later sacrificed (C4 & C5), pronounced ovarian growth (E-H type ovaries) was usually restricted to relatively young females (see below). These observations strongly suggest that *P. micans* females are totipotent in early adulthood.

In all nests where it was tracked, young wasps built and very seldom foraged while old wasps foraged and sometimes built. This indicates an age-related change in worker tasks (temporal

polyethism). Due to continuous envelope removal, pulp was by far the most common material delivered to the nest. Although task-allocation was not an emphasis of this study, it was evident that some foragers specialized in the collection of particular materials over a period of days while others were flexible.

On the day following the removal of the last queen of a nest, particular individuals were targeted for attack and were temporarily banished from the colony (Colonies 1, 4, 5 and 8). Those who were attacked found refuge off the nest (e.g., resting in isolation on the surrounding foliage) and intermittently attempted to return to their colony, where they were met by an assembly of patrolling workers. Upon landing, they were bitten and pinned down to the comb (Figure 4) while receiving mock stings from some of the aggressors. In three instances I observed a female who flew to the nest, endured and charged through the attacks, and disappeared inside the nest. I never observed a fatal or serious injury (e.g., a clipped wing) as a result of these attacks. The outbreak of worker aggression persisted over a period of 1 (Colony 5) to 3 days (Colony 4) after queen removal (Colonies1 and 8 were not observed beyond the day following queen removal). The onset of worker-worker aggression was observed to occur on the day of queen removal in Colony 5, where indiscriminant fighting (attackers quickly became attackees and vice versa, a situation that I will call a “mêlée”) broke out within a few hours of the loss of the last queen. All 10 aggressors sacrificed had filamentous ovaries. Whether this queenless mêlée preceded the inception of targeted attacks in other nests is not known.

Ovarian status

In colonies where all queens were removed (Colonies 1, 4, 5 & 8), those females who received attacks were relatively young (Figure 3) and, with exception of one female, had non-filamentous (i.e., ovaries sizes B-E of Fig. 2) ovaries (Figure 5). The attackers were relatively old (Figure 3), and every attacker sacrificed had filamentous (ovaries size A of Fig. 2) ovaries (Figure 5). A week following the elimination of the last queen, Colonies 4 and 5 were sacrificed. In both nests,

some paint-marked attackees were found inside, a group of females whom I will refer to as 'accepted'. In Colony 4, all accepted females had comparable or significantly larger ovaries (ovaries sizes B-H) than the attackees sacrificed in the days following queen removal (n=5). In contrast, accepted females from Colony 5 did not have larger ovaries than the attackees (n=6), and three marked females (A-C type ovaries) were observed to work (e.g., adding pulp to the nest envelope) only 3 days after the queen was removed.

Females who were attacked were not the only wasps to respond with ovarian growth once the last queen was removed. Within both Colonies 4 and 5, there were many females which had developing oocytes, and some females, including former builders, had very advanced ovaries. I refer to all females found within the nests as 'inside' wasps, irrespective of ovarian status or age (i.e., inside wasps were comprised of putative workers as well as potential reproductives) (Figure 5). Because I did not maintain constant surveillance of the nest following queen removal (due to the requisite wasp bleeding and dissection procedures performed while some of the attacks were taking place), it is likely that at least some females with maturing oocytes were attacked but not marked.

JH titers

Lone queens from monarchic nest had much higher JH titers than their worker counterparts in both colonies studied (Figure 6B). In the oligarchic nests, JH titers did not differ between queens and workers (Figure 6A). Yet the last queen removed, who reigned alone for 3 or 4 days before being sacrificed, had the highest JH titer among the queens removed from these nests (Figure 6A, see Ω for Colony 5 & 8). The eggless queen from the pre-swarming colony of Colony 7 (see above) had a low JH titer (1.3 pg/µl), comparable to that of a normal queen taken a few days later (2.2 pg/µl) and within the range of 6 workers sacrificed during that week before they swarmed (data not shown). Swarming queens consistently showed higher JH titers than workers constructing new cells in 3/3 colonies, although the difference was significant only for

Colony 7 (n=21, P=0.0003) and Colony 9 (n=8, P=0.03) but not for Colony 10 (n=13, P=0.09) (Figure 6C). Females from Colonies 9 and 10 were sampled within a month of one another (Table 1) and the radioimmunoassays were run together. When these data are combined, the difference in JH titer between queens and cell builders is significant (n=21, P=0.005). Workers from these colonies consisted of builders and foragers for Colonies 1 and 2, females who both built and foraged for pulp in Colony 6, and only builders for Colonies 4, 5, and 8-10.

Colony 2 contained workers which spanned all ages, and all non-queens had filamentous ovaries. JH titers were lower in builders and young females found within the nest interior than in the distinctly older foragers of pulp and liquid (n=24, P=0.004) (Figure 7). In contrast, young builders and relatively old foragers from Colony 1 showed no difference in JH titer; all workers had a JH titer below 3.5 pg/µl (n=16). In nests where sampled queenright workers enjoyed some ovarian growth (Colonies 1, 4, 5 and 8), JH titers were virtually identical (medians within 1 pg/µl) among workers possessing A or B-D stage ovaries (total n=41).

One day following final queen removal, JH titers were high in attacked, relatively young females possessing partially developed ovaries, while titers remained low in old, ovary-depleted aggressors (Figure 8). In 3 of the 4 colonies, there was a significant difference in JH titer between attackees and attackers [Colonies (1) n=7, P=0.08; (4) n=11, P=0.008; (5) n=10, P=0.01; (8) n=46, 0.001)]. The lack of a significant difference in Colony 1 is probably due to the small sample size of attackers (n=2). In support of this hypothesis, JH titers were significantly higher in attackees than in queenright workers sacrificed alongside the last remaining queen(s) in all 4 colonies [Colonies (1) n=21, P=0.007; (4) n=11, P=0.008; (5) n=23, P=0.0009; (8) n=41, P=0.003). There was no difference in JH titer between attackers and queenright workers in these colonies. Also, the mêlée group from Colony 5 had significantly lower JH titers than the attackees (n=13, P=0.03) and higher than queenright workers (n=26, P=0.0006) but no

difference from the attackers (Figure 8B). In 3 of the 4 colonies (Colony 1 was the exception), the JH titer showed no correlation with oocyte length in the day 1 attackees (data not shown).

In Colony 4, where attacks persisted for 3 days, JH titers declined in the attackees by day 3 (Figure 8A). In Colony 5, where attacks were less severe and lasted only one day, marked attackees were observed adding pulp to the nest on day 3 following queen elimination. These females had lower JH titers than fellow attackees who were sacrificed on the first day (Figure 8B).

Attackees who were eventually accepted back into the nest and captured 7 days after queen removal had low JH titers in Colony 4 ('Accepted' females in Fig.8A) and a great range of titers in Colony 5 (Fig. 8B). In neither case did JH titers correlate with oocyte length. An unmarked female with a swollen gaster from Colony 4 had well developed queen-like ovaries and a very high JH titer (Figure 8A). In the attempt to replicate this assay, 28 unmarked females from Colony 5 were bled for JH-RIA. No female with comparable ovaries was discovered during this session, but even among those females with developing ovaries, there was no correlation between JH titers and oocyte length.

Ecdysteroids

The ovaries of queens contain considerably more ecdysteroids than those of workers (e.g., Colony 2 of Figure 9A1). A comparison of the ovaries of queenless attackees of Colony 4 (ovaries size A-D), attackers of Colony 4 (ovaries size A) and queenright workers of Colony 2 (ovaries size A) showed no differences in ecdysteroid content (Fig. 9A2). Ovarian ecdysteroid content was not correlated with oocyte length in the attackees. The ovaries of both attackees and attackers contained less ecdysteroids than egg-bearing ovaries from the four accepted females (i.e., former attackees) (vs. attackers, n=16, $P<0.01$; vs. attackees, n=23, $P=0.004$; vs. workers, n=20, $P=0.003$) (Fig. 9A2). RIA analysis of partially developed ovaries (sizes B-D) from

workers, attackees and accepted females from Colony 5 showed no detectable amounts of ecdysteroid (n=8). Only putatively vitellogenic ovaries (sizes E-G) contained ecdysteroids (n=4), ranging from 2 to 92 pg (mean=45 pg). These data suggest that ovarian ecdysteroid production is increased at the onset of vitellogenin uptake by the oocyte.

Ovarian ecdysteroids were significantly correlated with ovary size in queens (ANOVA, P=0.001) (Figure 9B). The ovaries of the last queen remaining in Colony 5 contained more ecdysteroids than would be expected based on the size of her ovaries (Figure 9B, see Ω's). Similarly, the smaller ovaries of the new queen from Colony 4 contained significantly more ecdysteroids than the larger ones of Colony 2 queens (Figure 9A1).

Figure 9C shows the ecdysteroid titers in the hemolymph based on two separate RIAs. In Colony 2, the queens had higher ecdysteroid titers than the workers (n=23, P=0.02). For the 3 other colonies (6, 9, and 10), the ecdysteroid titers were low and there was no significant difference between the queens and workers. There was no significant correlation between hemolymph ecdysteroid titers and ovarian ecdysteroids or ovary size in queens of Colony 2 (n=10) and pooled queens from Colonies 6, 9 and 10 (n=14). Finally, there was no significant correlation between JH and ecdysteroid titers in queens who were bled for both hormone measurements (Colony 2 (n=11) or Colony 9 and 10 (n=9)).

Cuticular hydrocarbons

Analysis of the worker cuticular hydrocarbons (CHCs) identified 14 shared unambiguous hydrocarbons (Table 2), although many were always present at extremely low levels (0.0-0.5%). Twelve of 14 compounds were linear alkenes. The other two hydrocarbons were 3-methyl-C25 and an unidentified C27 alkene. In the text, these two compounds will be referred to as "C25a" and "C27z", respectively.

				QR All Colonies				*QR Colony 4*		*QR Colony 2*				*QL Colonies 4, 5 & 8*				*QL Colonies 4 & 8*			
				Queens		**Workers**		**Callows**		**Young**		**Old**		**Attackees**		**Attackers**		**Princesses**		**Workers**	
Peak	**hydrocarbon / component**	**HC abrev**	**Ret. Time**	**N=59**		**N=129**		**N=4**		**N=20**		**N=45**		**N=43**		**N=32**		**N=16**		**N=35**	
				mean	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD
1	Heneicosane	**C21**	18.07	**0.06**	0.02	**0.47**	0.48	**6.03**	0.59	**0.22**	0.07	**0.49**	0.34	**0.51**	0.20	**0.96**	0.66	**0.18**	0.06	**0.52**	0.68
2	Docosane	**C22**	20.83	**0.18**	0.18	**0.56**	0.61	**2.26**	0.15	**0.48**	0.36	**0.20**	0.11	**1.36**	0.92	**0.90**	0.53	**0.15**	0.04	**0.17**	0.10
3	Tricosane	**C23**	23.56	**2.25**	0.66	**7.02**	3.95	**54.44**	5.23	**4.46**	0.63	**7.70**	3.51	**8.38**	1.96	**9.36**	3.85	**3.45**	0.95	**6.56**	4.75
4	Tetracosane	**C24**	26.23	**0.93**	0.27	**1.32**	0.72	**2.15**	0.13	**1.43**	0.49	**0.98**	0.35	**2.76**	0.95	**1.76**	0.82	**1.26**	0.35	**0.87**	0.42
5	Pentacosane	**C25**	28.92	**42.53**	4.84	**23.72**	4.13	**22.43**	1.66	**24.12**	1.63	**22.75**	3.12	**25.67**	3.55	**24.20**	3.57	**33.41**	3.70	**24.11**	4.45
6	3-methyl-C25	**C25a**	30.00	**0.97**	0.67	**0.07**	0.05	**n.d.**	n/a	**0.04**	0.01	**0.05**	0.03	**0.05**	0.03	**0.05**	0.03	**0.23**	0.17	**0.03**	0.02
7	Hexacosane	**C26**	31.34	**5.32**	1.14	**3.42**	1.01	**1.90**	0.46	**4.24**	0.87	**3.25**	1.01	**4.82**	1.47	**3.32**	0.95	**4.48**	1.25	**3.21**	1.28
8	Z-(?)-heptacosene	**C27z**	33.65	**0.51**	0.36	**0.13**	0.05	**n.d.**	n/a	**0.12**	0.04	**0.12**	0.04	**0.08**	0.04	**0.08**	0.04	**0.50**	0.50	**0.11**	0.05
9	Heptacosane	**C27**	33.89	**44.57**	4.52	**57.74**	7.72	**8.29**	2.13	**57.75**	4.30	**58.49**	7.56	**44.92**	7.94	**52.31**	7.78	**51.72**	3.49	**60.41**	6.90
10	Octacosane	**C28**	36.14	**0.82**	0.73	**1.62**	1.23	**1.08**	0.34	**2.33**	1.21	**1.76**	1.37	**2.77**	1.85	**1.44**	0.69	**1.90**	1.40	**1.54**	1.61
11	Nonacosane	**C29**	38.42	**0.68**	0.68	**1.59**	1.45	**0.65**	0.30	**1.99**	1.11	**1.88**	1.45	**1.99**	1.53	**1.08**	0.56	**1.82**	1.25	**1.67**	1.65
12	Triacontane	**C30**	40.64	**0.52**	0.51	**0.98**	0.83	**0.48**	0.27	**1.31**	0.76	**0.98**	0.90	**1.48**	1.11	**0.75**	0.42	**0.92**	0.66	**0.78**	0.78
13	Hentriacontane	**C31**	42.77	**0.39**	0.39	**0.89**	1.16	**0.19**	0.10	**0.95**	0.57	**0.90**	0.90	*C31 & C32 were not detected in all of the above females and were therefore excluded*							
14	Docotriacontane	**C32**	44.99	**0.28**	0.26	**0.46**	0.44	**0.12**	0.04	**0.53**	0.33	**0.39**	0.38								

Table 2. Mean percentage of composition and standard deviation (SD) of cuticular hydrocarbons components in various types of females of *Polybia micans*. Colony(s) of origin noted on top. These data correspond to the bar graphs shown in Figures 12-14. Gray rows indicate components which are specific to queens and princesses (i.e., prospective reproductives on a queenless nest).

Queens vs. Workers

Queens (n=59) and workers (n=129) were pooled from all colonies from which epicuticular compounds were extracted (Colonies 2-8). Selected chromatograms for each phenotype are shown in Fig. 10). A stepwise discriminatory analysis (DA) based on 14 shared compounds significantly separated individuals according to their status (Global Wilks' $\lambda = 0.02733$; $F_{9,178}=703.77$; $p<0.001$), and obvious differences were observed in compound representation (Figure 11A). C21, C23, C24, C25, C25a, C26 and C27 alkanes were the main contributors of the separation (Table 3), and all except C27 were significantly different according to a *t*-test on log transformed percentages ($P<0.0001$). Neither the defeated queen from Colony 7 nor the lone queen from Colony 4 possessed a notably different CHC profile from other queens. Within all nests examined, C25a was invariably higher in queens than in workers. Indeed, queen status could be predicted based on the relative representation of this compound alone.

Groups (n)	C's	no. of HCs	Global Wilks' λ	mahalanobis distance	*F*-values	*p*-Level	matrix %	contributing compounds
Queen (60) x QR Worker (128)	2-8	14	0.027	162.02	703.77	<0.001	100	C21, C23, C24, C25, C25a, C26, C27,
Young (15) x Old Worker (45)	2	14	0.16	27.29	46.76	<0.001	98.3	C23, C25, C27
Attackee (32) x Attacker (43)	4,5,8	12	0.40	5.90	12.24	<0.001	89.3	C21, C23, C26, C27, C30
Prosp. Repro. (16) x QL Worker (35)	4,5	12	0.16	23.95	32.96	<0.001	98.0	C22, C23, C25, C25a, C27

Table 3. Summary of pairwise discriminatory analyses performed in this report. (C's=Colonies; Prosp. Repdo. = prospective reproductives; QL = queenless; QR = queenright)

Young vs. Old workers

The CHC profile of young wasps (cuticle score = 1-1.5; n=15) was compared to that of old workers (cuticle score = 4.5-5.0; n=45) in Colony 2 (where all non-queens had filamentous ovaries). Based on 15 shared compounds, the DA significantly separated young vs. old females (Global Wilks' λ = 0.15890; $F_{6,53}$=46.758; p<0.001). The most important compounds were C23, C25 and C27 (Table 3). Only C23 significantly differed between the age groups (P<0.0001, *t*-test) (Fig. 11B). The C25a alkane, which was distinguished in all queen samples (Table 1), was nearly undetectable young and old workers.

Four callow females from Colony 4, collected within three hours of eclosion from a comb maintained in the lab, showed a dramatically different CHC profile (Fig. 10 and 11B). The sample size was too small for a DA. Yet clearly the C23 alkane is predominant at this time, and then is at much lower levels in both young and old workers. C25a and C26z were not detected in these callow females (Table 2). The dramatic difference seen between callow females and young wasps with cuticle scores of 1 suggest that all young females collected from colonies in the field were at least several days old (cf. *Synoeca surinama*, Chapter 4).

Attackees vs. attackers (1-3 days after the final queen was removed)

Attackees and attackers from Colony 4 (n=29), Colony 5 (n=10) and Colony 8 (n=36) were pooled together, and the DA significantly separated individuals according to their role in the attacks (Global Model: Wilks' $\lambda = 0.40261$; $F = 12.24$; $p < 0.001$). The most important hydrocarbons for this separation were C21, C23, C26, C27 and C30 (Fig. 13A; Table 3). C23 ($P<0.05$), C26 ($P=0.01$) and C27 ($P<0.0001$) significantly differed between attackees and attackers (Figure 10C1).

Attacks persisted for 3 days in Colony 4, providing an opportunity to see how the CHC profile changed in potential reproductives (i.e., attackees) in the first three days following queen removal (Figure 13B). C25 and C25a, both queen-associated compounds, show a slight rise in the attackees. Contrary to the discriminatory analysis above, C26 and C27 did not show obvious differences for Colony 4, probably reflecting variability in CHC signaling between nests.

When young and old workers from Colony 2 (non-callows from Figure 11B) were added to the discriminatory analysis, the attackees showed a shift in the same direction from their attackers as did young females from their older nest mates (Figure 12C), suggesting that age, at least in part, is a contributor to the differences between attackees and attackers.

Thus, the attackees appear to reflect their youth in their CHC profile, and queen-associated compounds show a perceptible rise in attackees within a few days of queen removal. Whether young potential reproductives show an *immediate* shift in their CHC profile in response to queen removal – which could be used by the attackers as a signal for policing – was not explicitly tested. It is clear, however, that if the CHC profile of young potential reproductives does change to signal new information as potential reproductives transition from a queenright to queenless nest, the shift is very subtle.

Prospective reproductives vs. putative workers in a queenless nest (7 days after ultimate queen removal)

Females found inside the nests on day 7 after final queen removal were grouped based on ovarian state. Those females with developing ovaries, from stage D-I (n=16), were considered prospective reproductives (PR) and were separated from females with A-C ovaries (n=35) who were presumptive workers (PW). Data from Colony 4 (n=19) & 5 (n=32) were pooled, and 12 shared compounds were included in the DA which showed a significant separation based on ovary state (Global Model: Wilks' λ = 0.15708; $F_{7,43}$= 32.96; P< 0.001). Main contributors were C23, C24, C25a, C26 and C28. Of these, only C23 (P<0.0001), C24 (P=0.03) and C25a (P<0.0001) showed a significant difference (*t*-test). Figure 13 includes the CHC profile of queens from Colony 4 (n=1) & 5 (n=5). The C25a alkane is present in both queens and the potential reproductives, but not in the putative workers (Fig. 13).

Might the difference in the CHC profile between prospective reproductives and putative workers be related to age? To investigate this, I grouped the above samples according to ovary size (A vs. B-C vs. D-H) (Fig. 14A) or cuticular age [2.5-3.0 (younger) vs. 3.5-4.0 (mid) vs. 4.5-5.0 (older)] (Fig. 14B) and performed DAs using the same 12 compounds. Grouping by ovary size clearly resulted in better separation (Wilks' λ = 0.06917 approx.; $F_{18,80}$=12.455; P< 0.0001; classification matrix=96%) than by relative age (Wilks' λ = 0.51736; $F_{8,90}$=4.3907; P< .0002; classification matrix=67%).

A DA based on pooled data from queens, queenright workers, attackees, attackers, prospective reproductives and queenless workers from Colonies 4, 5 & 8 revealed that prospective reproductives show a clear shift toward a queen-like CHC profile (Fig. 15C), confirming the pattern seen in Fig. 13. Also, those females without a queen for 7 days, regardless of status or

group, separate from the cluster of queenright workers, queenless attackees and queenless attackers (Fig. 14C).

Together the above analyses indicate that the CHC C25a is always present in the queen, albeit at low concentrations. Figure 16 shows that the level of C25a is always higher in queens than workers (5 of 5 colonies) and, on average, higher in potential replacement reproductives than in queenless workers of the same nest (2 of 2 colonies).

Discussion

Juvenile hormone and queen behavior in wasps

In primitively eusocial wasp societies, where a single queen dominates the colony by force (e.g., most *Polistes* sp.), JH is important for sustaining reproductive growth and dominance-related aggression (Roseler et al., 1980; Roseler et al., 1984; Strambi, 1990; Tibbetts et al., 2011; Tibbetts and Izzo, 2009). Just as testosterone in many vertebrates (Wingfield et al., 1990), JH is up-regulated in periods of social competition among potential reproductives (Tibbetts and Huang, 2010). In Epiponini wasps, where many queens are tolerated, reproductive competition persists throughout the nest cycle, until only one queen remains, at least on smaller nests (cyclical oligarchy) (West-Eberhard, 1978b).

In the present study, we show that in founding colonies of *P. micans*, queens had higher JH titers than workers, consistent with our hypothesis. Yet in established nests undergoing comb construction, queens did not have higher JH titers than their worker counterparts. In both types of nests, many empty cells are made available, so competition among queens is expected to be high. What could explain the difference in endocrine activity between these types of nests? Swarming behavior, where members of a colony abandon a nest in favor of founding a new one, is likely an endocrine-mediated event, and JH titers are known to modulate relocation behaviors

(e.g., migration) in other insects (Dingle and Winchell, 1997). Therefore, the most direct data set for assessing a role of JH in reproductive competition comes from the established colonies, which also included larger sample sizes. It thus appears that JH is not important for reproductive competition per se in *P. micans*.

Still more surprising, monarchic queens of *P. micans* had much higher JH titers than workers of the same nest. Despite the lack of immediate competition, these lone queens were noted for the intensity of their worker-directed abdomen bending, a behavior rarely observed in multi-queen nests, where queens appear to communicate dominance by ignoring the workers' queen dance (i.e., there is no requisite to stop and bend). Although queen bending was rarely noted in queen-worker interactions on oligarchic colonies, the architecture of the nest precluded observation of queen-queen interactions where abdomen bending is known to occur in other Epiponini wasps (Nascimento et al., 2004; West-Eberhard, 1977). While high JH titers are also observed in queens of *Polistes* (Giray et al., 2005), who are by rule monarchic, the social organization of these colonies is very different. *Polistes* societies are based on a linear dominance hierarchy which queens must control on their own. Monarchic swarm founding colonies are not hierarchally-based, and if future queens do emerge, they fission (i.e., swarm away) from the colony (West-Eberhard, 1978b). Thus, in *P. micans* queens, JH titers are associated with ritualized displays of aggression but are not necessary for sustaining reproduction or reproductive competition.

The widely conserved gonadotropic role for JH in solitary and primitively eusocial insects (e.g., *Polistes*, *Ropalidia*, *Bombus*) has been lost in adult queens of honeybees and stingless bees (Hartfelder and Emlen, 2011). Pyriproxyfen (JH mimic) application has been shown to actually reduce fertility in dominant wasps and produce a worker-like CHC profile in queens of a secondarily queenless species of ant (Cuvillier-Hot et al., 2004). In line with these results in highly eusocial insects, we show a reduced dependence on JH for sustaining reproductive

development in queens of *Polybia*, the most derived genus of eusocial wasps (Pickett and Carpenter, 2010).

Hormonal control of reproductive maturation in *P. micans*

There is little evidence presented here to suggest a role for JH in sustaining ovarian maturation among putative future queens or even queenright workers. An exception to this was the new queen of Colony 4 who had a high JH titer and large ovaries which dwarfed those of other prospective reproductives who had low JH titers. Like monarchic queens of *P. micans*, she was developing her ovaries in a colony without firmly established queens (although there were other emerging queens present). One hypothesis is that the absence of a signaling or inhibitory queen pheromone (perhaps from glands in the head; see West-Eberhard, 1977) might lead to unchecked, rapid ovarian growth. In support of this, the ecdysteroid content of ovaries from the last standing queen of Colony 5 and the new queen from Colony 4 were much higher than expected based on ovarian size. Might this boost of ovarian ecdysteroids, an indication of maturing oocytes, be somehow facilitated by high levels of JH? Obviously, more hormone measurements, coupled with JH application experiments, will be necessary to test augmenting functions of JH in potential as well as actual reproductives.

In *Polistes*, ecdysteroids produced in the ovaries are released in the hemolymph and have been demonstrated to be important for establishing dominance (Roseler et al., 1985; Strambi, 1990). In some insects such as mosquitos, ecdysteroids have taken over gonadotropic functions once regulated by JH (Nijhout, 1994; Wyatt and Davey, 1996). I thus investigated whether ecdysteroids in the hemolymph correlate with ovarian status and/or regulate dominance behavior. I show here that ecdysteroid titers, although detected in some queens usually at low levels, show an extreme range of values and/or show no difference to those workers who possess filamentous ovaries. Ovarian ecdysteroids were not correlated with hemolymph ecdysteroid titers, unlike other social Hymenoptera where circulating ecdysteroids have been

shown to be important (Geva et al., 2005; Roseler et al., 1985). In *Polistes dominulus* (formerly *gallicus*), hemolymph titers of foundresses are comparable to the range of values seen in Colony 2 of *P. micans*. However, ovariectomized foundresses had very low ecdysteroid titers and were within range of the other three colonies of *P. micans* (Roseler et al., 1985). I am therefore skeptical that hemolymph ecdysteroids are biologically important for *P. micans*.

Juvenile hormone and social interactions

Although JH does not appear to be very important for regulating basic queen physiology, JH titers surged in young potential reproductives a day after all queens were eliminated from the nest. In *Polistes*, competing replacement queens sustain JH high titers (eclipsing that of the original queen) (Tibbetts and Huang, 2010), but in *P. micans* the elevated titers declined by the third day and normally remained low even as females develop eggs. The transient spike of JH occurred in response to social upheaval whereby an opportunity for direct reproduction could be realized. This opportunity, however, was concomitant with the reception of attacks from older, effectively sterile females.

Whether JH rises or falls in response to a stressor is species-dependent in insects. When the context is explored, it is clear that JH is modifying development in an adaptive manner. For instance, in moth caterpillars, JH increases in response to starvation (Riddiford, 1980) and other stressors (Tauchman et al., 2007). This makes sense because caterpillars may need to prolong the larval stage in hopes of finding more food before risking metamorphosis (Riddiford, 1980). Also, stress by way of crowding in termites leads to an increase in JH levels (Mao and Henderson, 2010). Termites are by and large a colony of ontogenetically totipotent immatures, and a high JH level induces soldier differentiation (Korb and Hartfelder, 2008), indicating that a rise in JH in this context is a social adaptation. Indeed, the addition of soldiers to crowded conditions led to a decrease in the levels of JH in workers (Mao and Henderson, 2010). Thus, in

immature insects, JH is not a “stress” hormone per se, but leads to a physiologically appropriate (i.e., adaptive) response to stress.

In non-termite orthopteroid insects, including *Locusta migratoria* and *Schistocerca gregaria*, crowding stress leads to a *decrease* in JH levels (measured in vitro) (Dale and Tobe, 1986; Injeyan and Tobe, 1981). In adult *Drosophila*, both JH and 20E, via octopamine, are thought to be involved in non-specific stress responses (Gruntenko and Rauschenbach, 2008). Essentially, unfavorable conditions (e.g., heat stress) led to decreased JH esterase activity (i.e., higher levels of JH) and an increase in 20E in both males and females. Honeybees show a JH response to being caged and cold anesthetized, considered “high” and “low” stressors, respectively (Lin et al., 2004). In both conditions, nurse bees showed a significant rise in JH titers after only 30 minutes and >100 fold difference after 24 hours in some cases. Conversely, JH titers rose much more slowly in foragers and only in some colonies. Indeed, not all hives showed a rise in JH titers in response to stress, and in some cases the response was reversed, even in active caged bees, results the authors note as evidence that JH is not a “stress” hormone in honeybees (Lin et al., 2004). In the present study, cold anesthetized *P. micans* did not show a trend toward increasing JH titers over a 4 hour period, providing some evidence that JH may not be involved in “low” or “unconscious” stress. However, I did exclude compromised JH titer data from Colony 3 in this report because one of two queens was mishandled in an attempted collection. This presumed “high” stressed queen fled the colony and returned 30 minutes later. She had the highest JH titer of the 22 females collected from Colony 3. With regards to intranest causes of stress, in *S. surinama*, a fellow swarm founding wasp, attacked females with reproductive potential do *not* show a rise in JH; rather, they show a marked reduction or squelching of JH titers (Chapter 4). Thus, it remains unclear if JH is consistently involved in the stress response for *P. micans*.

The best evidence that JH was not acting as a stress hormone in the attackees of *P. micans* is that JH titers did not remain high throughout the period of attacks. In Colony 4, where attacks persisted for three days, JH titers were low in all females still receiving attacks on the third day. Thus, if JH is a stress-response hormone in *P. micans*, it must be activating downstream factors to cope with the on-going attacks. This would be a curious mechanism since stress hormones typically stay high during transitory stress. For example, in social vertebrates, stress hormones rise in challenging, instable situations and remain high for the duration of the event (e.g., mating opportunities) before returning to normal levels (Sapolsky, 1992).

Although I cannot rule out the possibility that JH initiates a physiological cascade for coping with social stress, I hypothesize that JH provides the ovaries of potential reproductives with competence for further development, a known physiological function of JH in numerous other insects (Wyatt and Davey, 1996). For example, in *Manduca sexta*, JH is not necessary for the onset of vitellogenin production or uptake but is necessary for progression beyond the 1 mm stage which requires both more Vg uptake and hydration before chorionation begins. If JH does not appear, eventually the 1 mm oocyte is resorbed and the next one in the ovariole progresses to the 1 mm stage (Nijhout and Riddiford, 1979). A similar mechanism may be in place in *P. micans*. In support of a reproductive role for JH in *P. micans*, JH does associate with actual reproductives of *P. micans* in certain contexts (e.g., lone queens, swarming queens, a replacement queen with massive ovaries), suggesting JH has at least an auxiliary role in queen physiology or behavior, enigmatic as it is. Finally, JH is associated with reproduction in *S. surinama* (Chapter 4), and so a link between JH and queenhood is conserved in at least some swarm-founding wasps.

Whether the rise in JH affects the CHC profile of the attackees is difficult to determine since relatively young females with partially developed ovaries already have a somewhat distinct chemical profile from older, attacking workers. Indeed, chemical signatures of age and/or

ovarian condition alone may be sufficient for attackers to detect and police potential reproductive when the colony becomes queenless. In a closely related species, *Polybia occidentalis*, methoprene was applied to young totipotent individuals, and precocious behaviors were observed (O'Donnell and Jeanne, 1993). There was no report of these females receiving attacks (cf. *S. surinama*; Chapter 4), suggesting that their CHC profile did not shift toward that of a potential reproductive. The lack of a JH-mediated CHC fertility signal may also be the case for *P. micans* since the distinction in CHC profile between attackees and attackers on the day after queen removal was small, although queen-associated hydrocarbons (e.g., C25, C25a) did increase in the attackees thereafter. These observations, coupled with the evidence of low JH titers in queens and prospective reproductives with maturing oocytes, suggest that JH is not involved in fertility signaling in attackees. Instead, I hypothesize that workers, in recognizing the absence of the final queen, are compelled to attack potential reproductives based on pre-existing cues for ovarian and/or age as conveyed through the epicuticle. Also, it is possible that JH led to the production of pheromones not from the cuticle but from specialized glands, a possibility not tested here.

Broadly, the attacks by workers appear to be a mechanism of queen selection from a pool of potential reproductives. Some attackees became actual reproductives while others became workers or reverted back to working. It is unclear if all queenless females with partially developed ovaries are attacked and/or whether some are spared and ‘chosen’ as future queens. The attacks may serve to counteract the hypothesized JH-mediated ovarian maturation, as many attackees did not have vitellogenic ovaries seven days after final queen removal. Attackees who endured and produced egg-bearing ovaries may have been in better condition to overcome the bouts of aggression. Indeed, observations of females flying onto and charging into the nest suggest that some attackees were more resilient than others, but it could be that they did not elicit fierce attacks (e.g., their status was ambiguous). Obviously, more work needs

to be done to determine the social as well as endocrine mediated mechanisms of queen succession in *P. micans*.

Juvenile hormone and foraging behavior in wasps

We show here, for the first time in any wasp, that JH titers were significantly lower in young females than older foragers (Colony 2). However, in other queenright nests, foragers had very low JH titers, although there was great range (and no pattern) of JH titers in females with filamentous ovaries from the queenless nests of Colony 5. It must be noted that the differences observed in Colony 2 are not nearly as dramatic as that observed in honeybees, which typically show a 10 fold difference in JH titer between nurses and foragers (Elekonich et al., 2001). Therefore, these results should be interpreted cautiously, as the difference observed here may not be biologically significant. It is worth noting that most of the evidence suggesting a role for JH in modulating age-related changes in behavior (temporal polyethism) is based on methoprene application experiments divorced from hormone measurement (O'Donnell and Jeanne, 1993; Shorter and Tibbetts, 2009), an approach which has received some criticism (Zera, 2007). When JH titers of foragers are determined, they are typically low and not distinguished from younger wasps (Tibbetts and Huang, 2010). These data could be reconciled if JH levels rise but then fall as workers transition into other tasks. On the other hand, Giray et al (2005) show that in *Polistes canadensis* (where guarding but not foraging was strongly age-related in their study population), JH is higher in guards while methoprene induces precocious guarding behaviors, indicating that JH does indeed regulate aspects of sequential task performance in wasps.

Cuticular hydrocarbon profiles

CHC profiles varied according to status, age and ovarian condition in *P. micans*. Here, we show that CHC profiles of actual or potential reproductives differ from their co-existing non-

reproductives. Since JH titers almost never correlate with ovarian condition in *P. micans*, a JH-mediated fertility signal in *P. micans* is not tenable. By contrast, in foundresses of *Polistes dominulus*, where JH titers, fecundity and CHC profiles are tightly correlated, JH has been hypothesized to directly regulate a female's CHC profile (Izzo et al., 2010).

C25a (3-methyl-C25) is a queen-specific compound. Within any given nest, C25a was always higher in queens and never overlapped the range observed in workers. As would be expected, prospective reproductives with developing oocytes showed an increase in this compound. Despite the strong association with queens, C25a is always present at very low levels (0.2-2%), but as has been shown in studies on floral odorants, sometimes the scarcest compounds are the most important (Riffell et al., 2009). Whether C25a represents a true pheromone cannot be determined at this time.

The CHC profile of newly emerged (i.e., callow) female wasps was dramatically different than that of *in situ* females, even those classified by cuticular age to be very young. Thus, the CHC profile of callow females must change very rapidly, as has observed in other social insects (ref). Young, non-callow wasps exhibit a distinct profile from markedly older wasps as well. This is not surprising since these females, like all freshly ecdysed insects, have soft cuticles which harden and sclerotize over hours or days.

Bringing it together: summary and concluding remarks

This is the first true endocrine study of a highly eusocial wasp, and there were many surprises. In direct contrast to the endocrinology of *Polistes*, JH is not involved in normal reproductive competition nor does it drive the reproductive physiology of queens or prospective queens, and, consequentially, appears not to be important for CHC fertility signaling. In fact, counter to all expectations based on a *Polistes* ground plan, JH titers elevated only in the *absence* of competition (monarchic colonies). Since JH has lost important reproductive, dominance, and

chemical signaling functions in *P. micans*, one might have expected circulating ecdysteroids, which appear to have JH-redundant functions in *Polistes* (Roseler, 1985; Roseler et al., 1985; Roseler et al., 1984), to have emerged as the principal regulators of reproduction, signaling and behavior in more derived wasps. This hypothesis is squarely rejected since hemolymph ecdysteroids are not consistently detected in *P. micans*. Despite sharing a common caste-based ancestor, Polistini and Epiponini wasps have, superficially at least, wildly different endocrine ground plans.

Looking deeper, there may be some putative synapomorphies in the endocrinology of *Polistes* and *P. micans*. Elevated levels of JH in single queens and emerging queens with large ovaries may augment reproductive growth, and so a gonadotropic function for JH may be preserved in some contexts. The most striking pattern of JH expression was seen in potential reproductives on queenless nests. In *Polistes*, the elevated levels are thought to be analogous to testosterone in vertebrates: they increase an individual's ability to compete in periods of instability (Tibbetts and Huang, 2010). In *P. micans*, JH may fuel dominance indirectly: only the strongest females can charge through their attackers and assert themselves as future queens. A key difference between these species, however, is the ~48 hour rise and fall JH of in *P. micans*. If JH primes the ovaries for maturation and increases a wasp's ability to become queen, then a conserved role for JH in social wasps will be apparent. However, a stress-induced response to attacks cannot be ruled out, and so future studies are required to elucidate the actual role of JH in this context. For example, are some future reproductives spared from attack, and do they also experience an initial or even sustained rise in JH following queen removal? Assays like this, coupled with JH and JHA treatments on the nest and in the laboratory will be essential for understanding the social biology of *P. micans*.

It is evident that the endocrinology of social wasps has undergone extensive remodeling. Fortunately, we will be able to track how these changes may have evolved by studying species

phylogenetically intermediate to *Polistes* and *Polybia* (Pickett and Carpenter, 2010). The Epiponini is comprised of hundreds of species with varying complexity and specializations which are begging for study (Jeanne, 1991). The behavior of astelocyttarous clade of wasps, which consists of *Metapolybia*, *Syneoca* and *Astelocea*, is well known due to their unique nest architecture which permit unimpeded observation of the entire colony (Nascimento et al., 2004; West-Eberhard, 1977, 1978b). In particular, *Synoeca* species are notorious for their large size and aggressiveness, a perfect candidate for hormone assays. I was fortunate to find a dozen nests of *Synoeca surinama* at my field site, and so the story of endocrine evolution in social wasps continues in Chapter 4.

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Figures

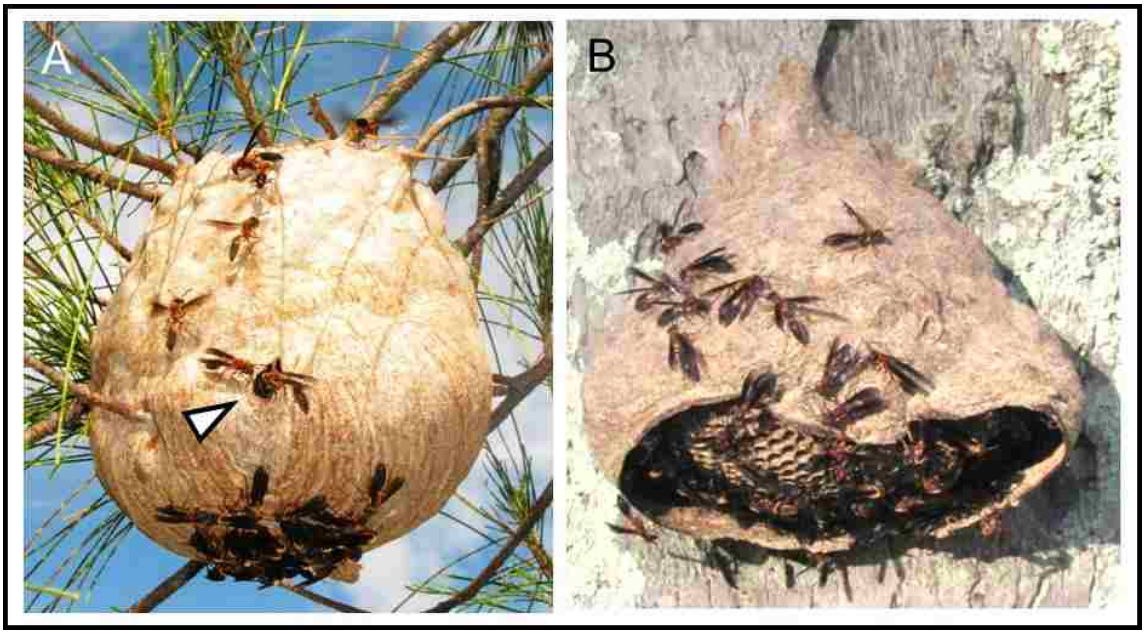


Figure 1: Nest architecture of *Polybia micans*. (A) A typical nest. Arrowhead indicates entrance/exit hole. The aggregation of workers outside the nest is often a foretelling of nest expansion. (B) Nest in a state of expansion.

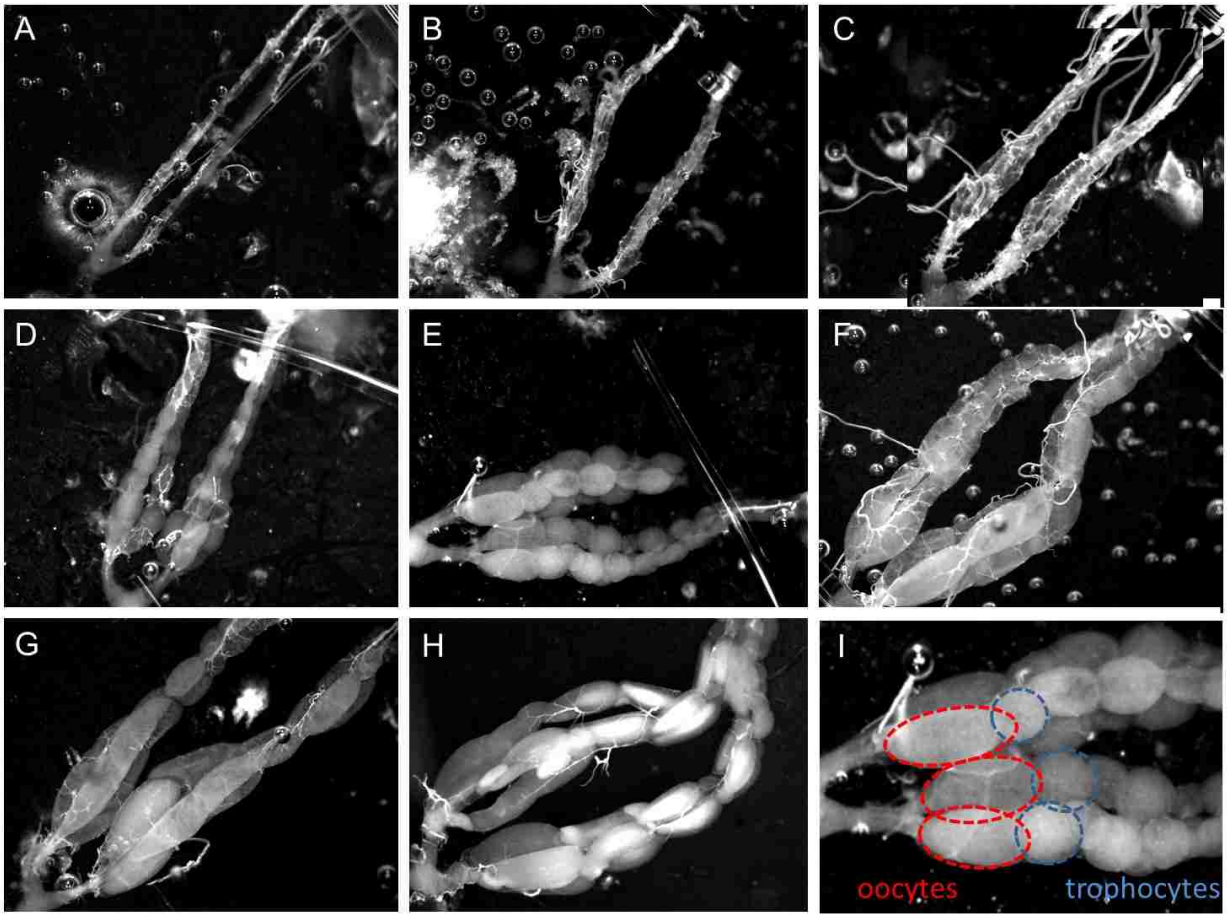


Figure 2. Score of ovarian status. (A) Filamentous ovaries. (B) Non-filamentous but irregular, slightly bulbous ovaroles. (C) Several residual or very small oocytes are discernible. (D) Oocytes and adjoining nurse cells are clearly evident and of comparable length. (E) Oocyte is elongated, possibly marking the onset of vitellogenin uptake. (F) Primary oocytes have absorbed most or all of the cytoplasm from the nurse cells. (G) 1- 3 chorinated oocytes are present. (H) Many eggs (i.e., queen-like). (I) Ovaries from box E with 3 of 6 primary oocytes (red) and adjoining trophocytes (blue) circled.

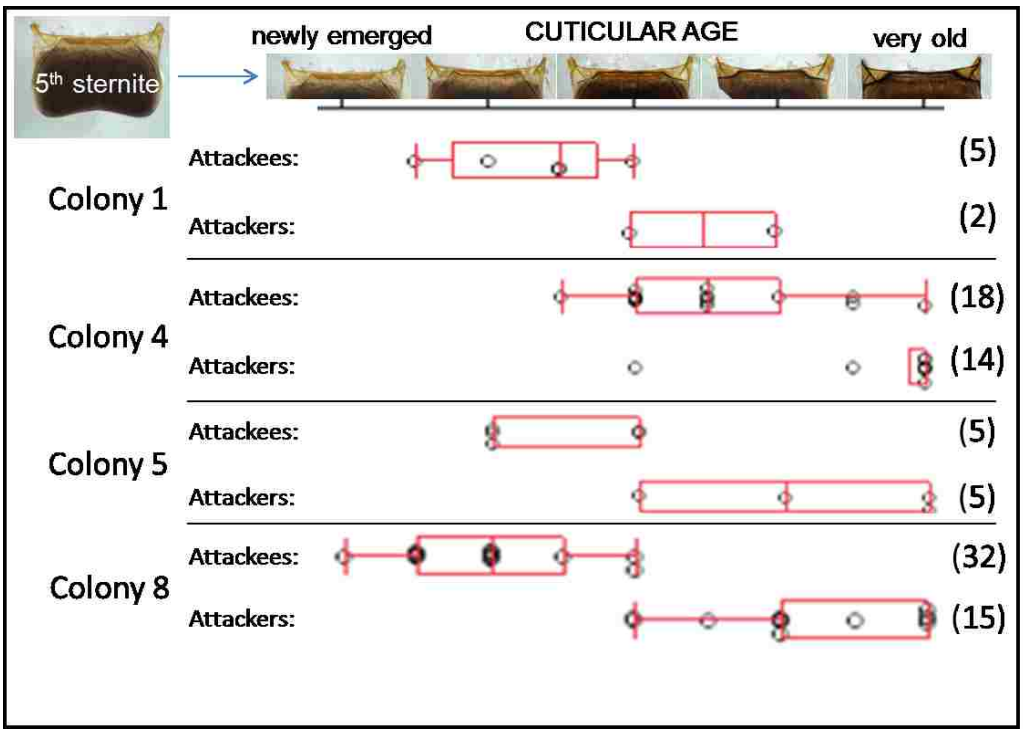


Figure 3. Relative age of attackees and attackers on queenless nests based on the analysis of apodeme darkening on the 5th sternite. Sample sizes are indicated in parentheses; boxes indicate the middle 50% of cuticle scores; whiskers indicate data within the 1.5 interquartile range.

Figure 4. One day after queen removal on a nest of *Polybia micans*. Arrow points to an 'attackee' who is bitten and pinned down to the comb by older workers ('attackers').

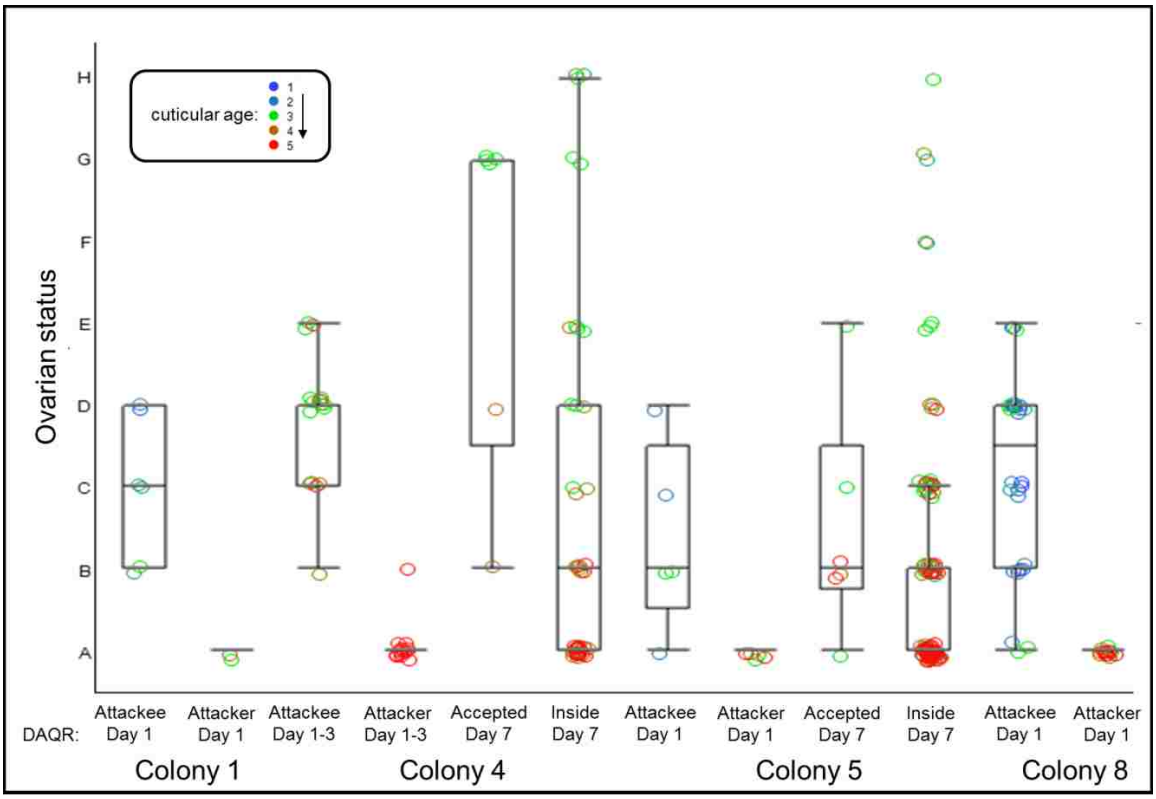


Figure 5. Ovarian status of females 1, 1-3 or 7 days after queen removal (DAQR) from 4 colonies. Ovarian stage was classified according to the index presented in Figure 2. Age estimates (in color) are estimates based on a cuticular character (see Fig. 3). Attackees have larger ovaries and are younger than their attackers. Some 'accepted' females, those who were attacked 1-3 DAQR but later were accepted back into the nest, had larger ovaries than those attacked in Colony 4 but not in Colony 5. Inside wasps are a sampling of females who were not observed to be attacked. Box plots show the middle 50% of ovary scores and the whiskers indicate the 1.5 interquartile range.

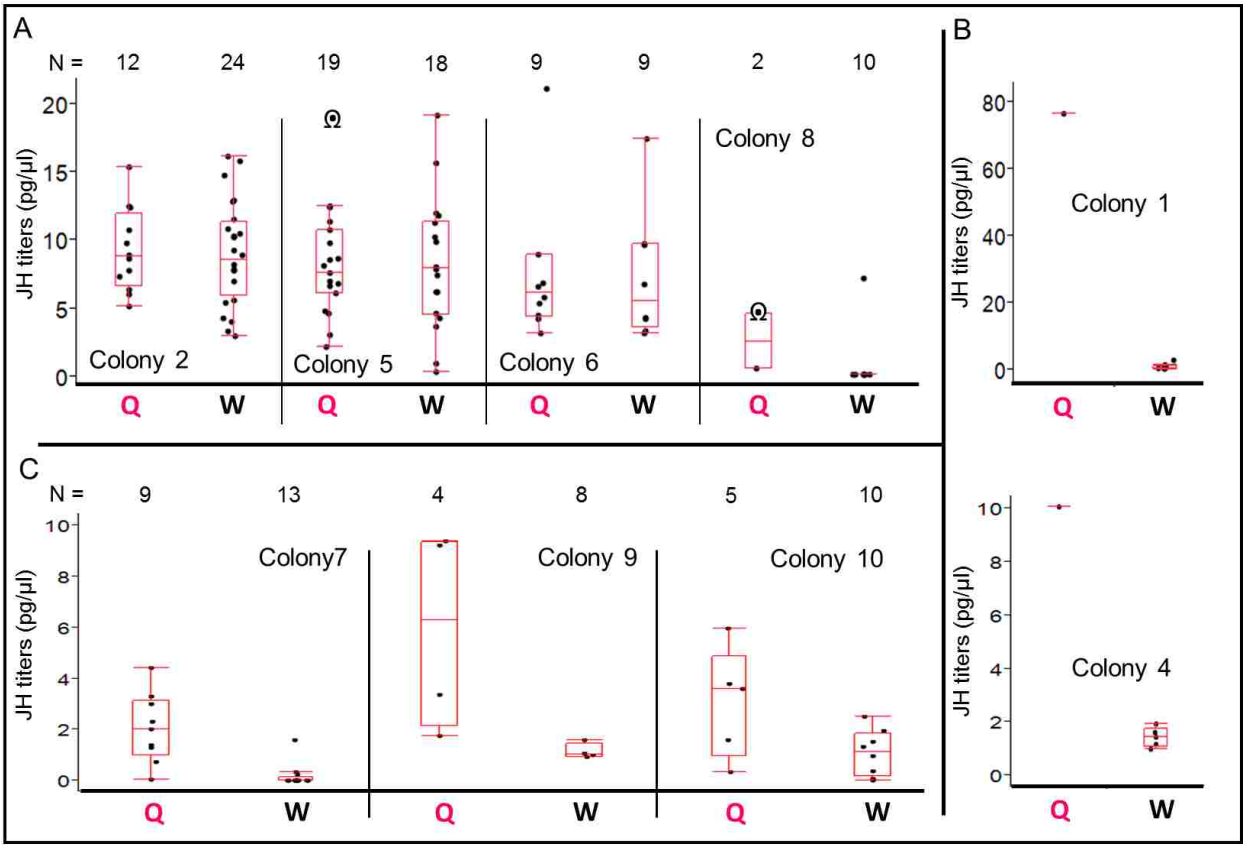


Figure 6. JH titers of queens and workers from (A) established oligarchical colonies, (B) established monarchical colonies and (C) founding colonies. (A) There are no significant differences between queens and workers. Ω indicates the last queen removed from Colony 5 & 8 (not all queens were removed from Colony 2 & 6). (B) Single rule queens have much higher JH than queenright workers. (C) A significant difference is observed in Colony 7 (P=0.0003) and Colony 9 (P=0.03) but not in Colony 10 (P=0.09).

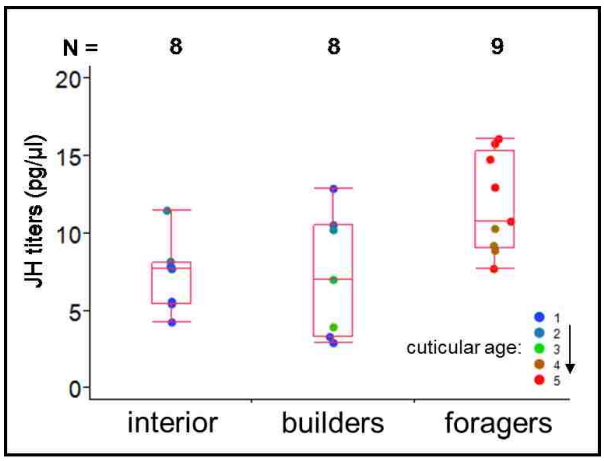


Figure 7. JH titers of inside wasps not yet observed to work (i.e., young pre-workers), builders and foragers (of pulp and liquid) from Colony 2. All females possessed filamentous ovaries. Age estimates (in color) are based on cuticular characters shown in Fig. 2.

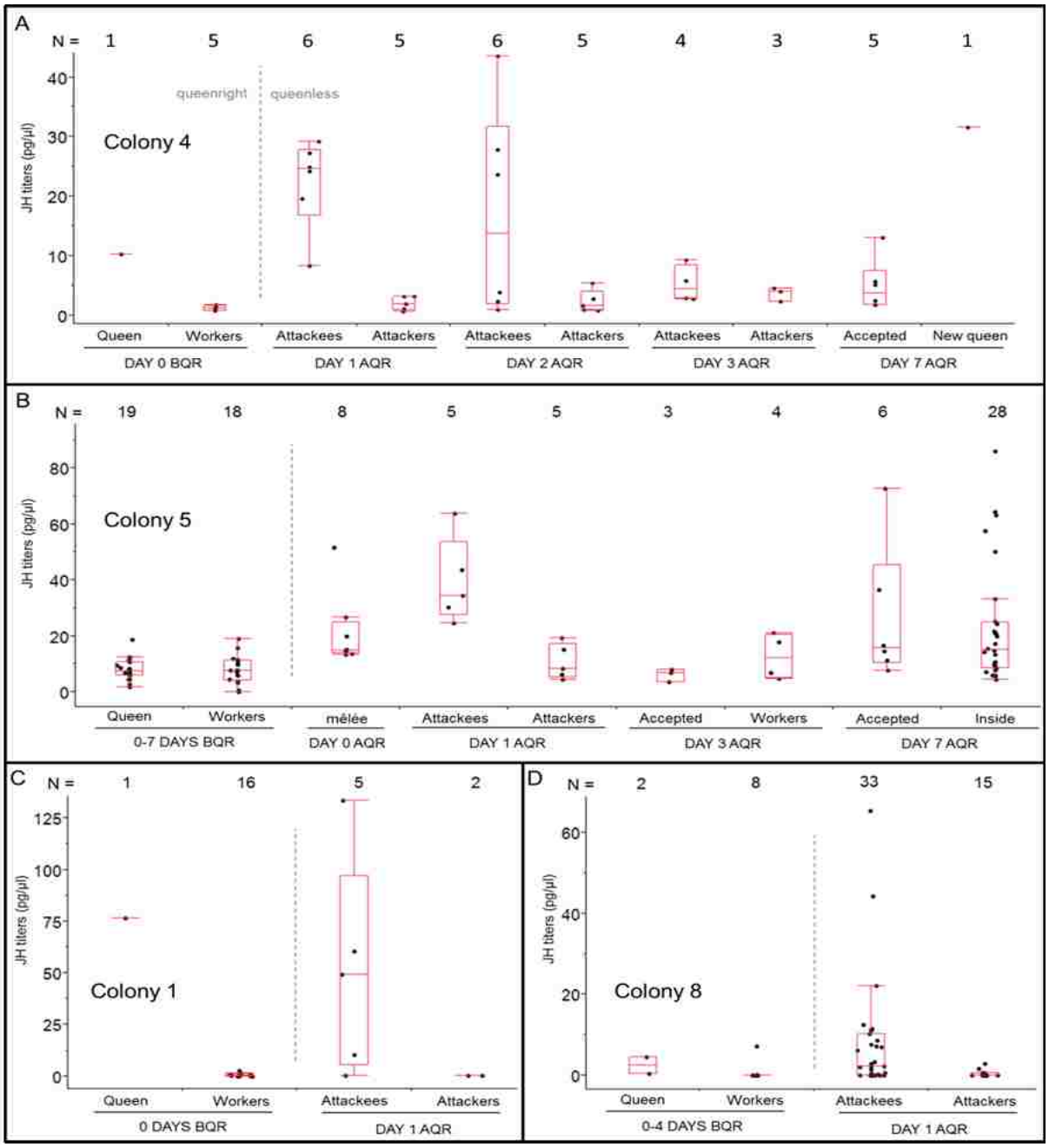


Figure 8. JH titers of females from queenless colonies (data from queens and queenright workers are shown for comparison). Day of sacrifice indicated with respect to queen removal (BQR = before queen removal; AQR = after queen removal). (A) Colony 4: Attackees from day 1 AQR had higher JH titers than attackers from day 1 AQR (P=0.008) and all other multiple sample groups. (B) Colony 5: Attackees from day 1 AQR had higher JH titers than attackers from day 1 AQR (P=0.01) and all other groups through day 3 AQR (P<0.03). Mêlée attackers had higher JH than queenright workers. (C) Colony 1: Attackees did not have higher JH titers than their attackers (but see text). (D) Colony 8: Attackees had significantly higher JH titers than their attackers (P=0.003).

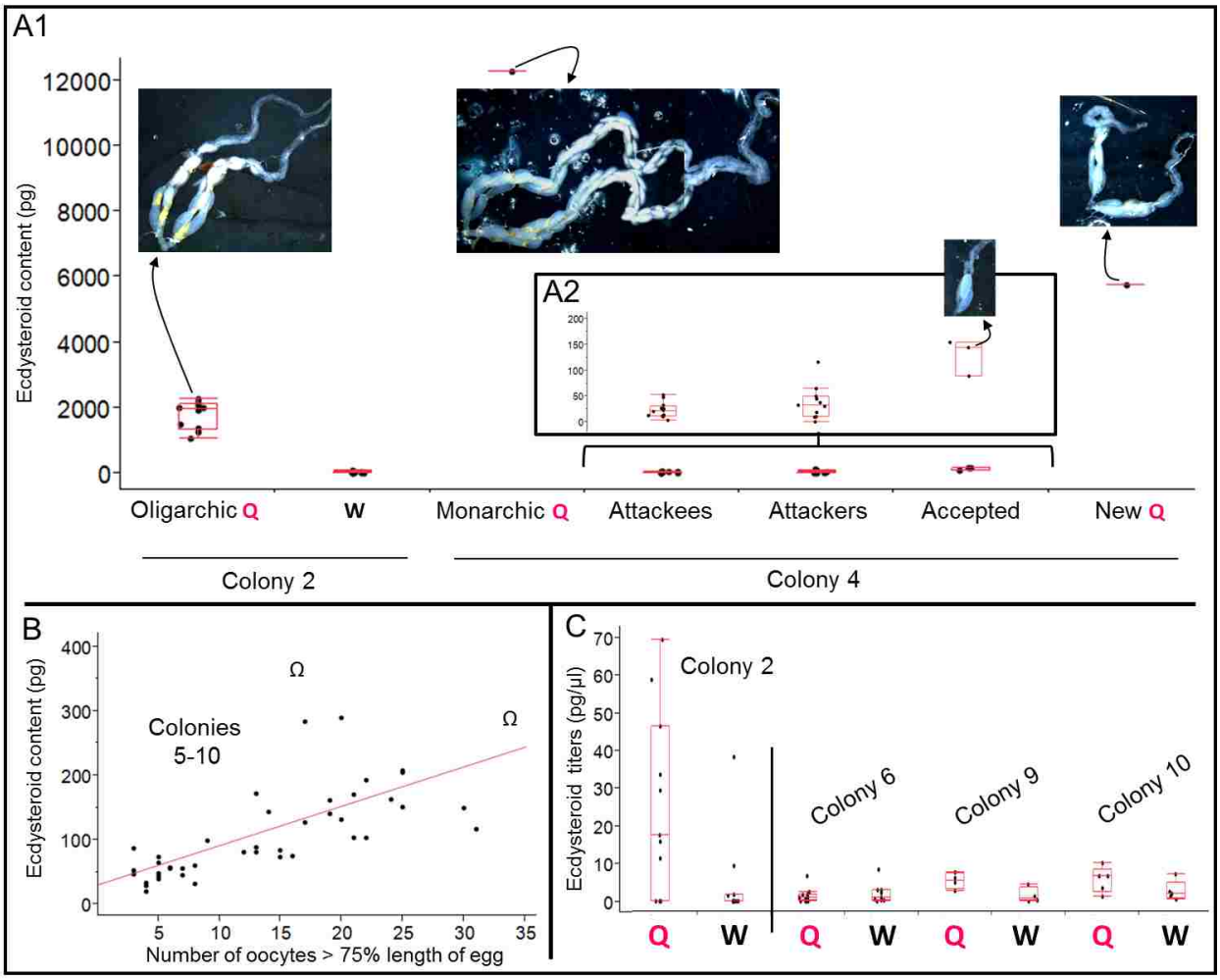


Figure 9. Ecdysteroids in *Polybia micans*. (A1) Ecdysteroid content of the ovaries among queens, workers and transitional types. Queens have much more ecdysteroids than their worker counterparts. Ecdysteroids were detected but scattered in queenright workers (Colony 2), (A2) attackees (Colony 4) and attackers (Colony 4). All groups had lower ecdysteroids than accepted females with 1-3 eggs (type G ovaries). (B) Ovarian ecdysteroids were significantly correlated with ovary size in queens (ANOVA, P=0.001). Ω indicate last queens removed from a nest. (C) Ecdysteroid titers of queens and workers from 4 colonies. In Colony 2, queens had higher ecdysteroid titers than workers (n=23, P=0.02). In a separate RIA, a statistically significant difference was found between founding queens and workers only when data from Colony 9 and Colony 10 were combined (P=0.017).

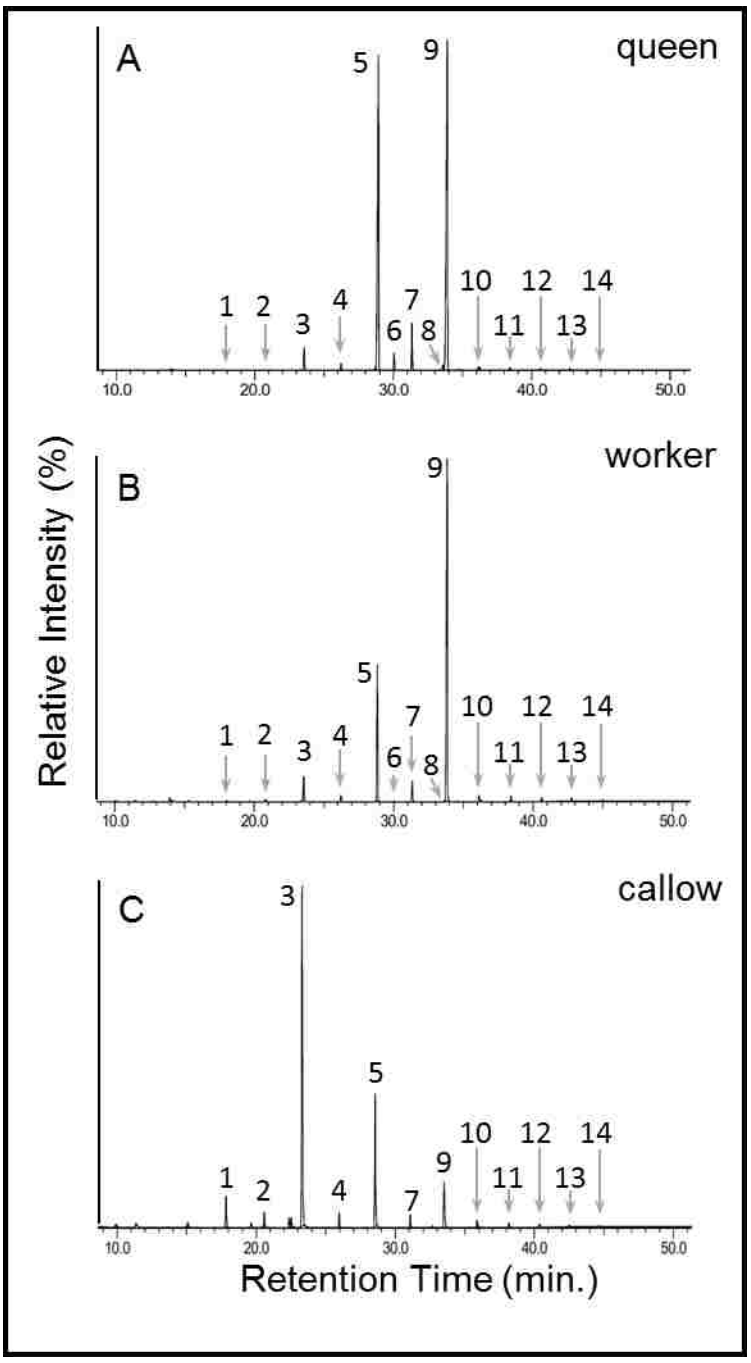


Figure 11. Chromatogram of whole-body extracts of queens, workers and callow females from *Polybia micans.* For compound numbering and identification, refer to Table 2.

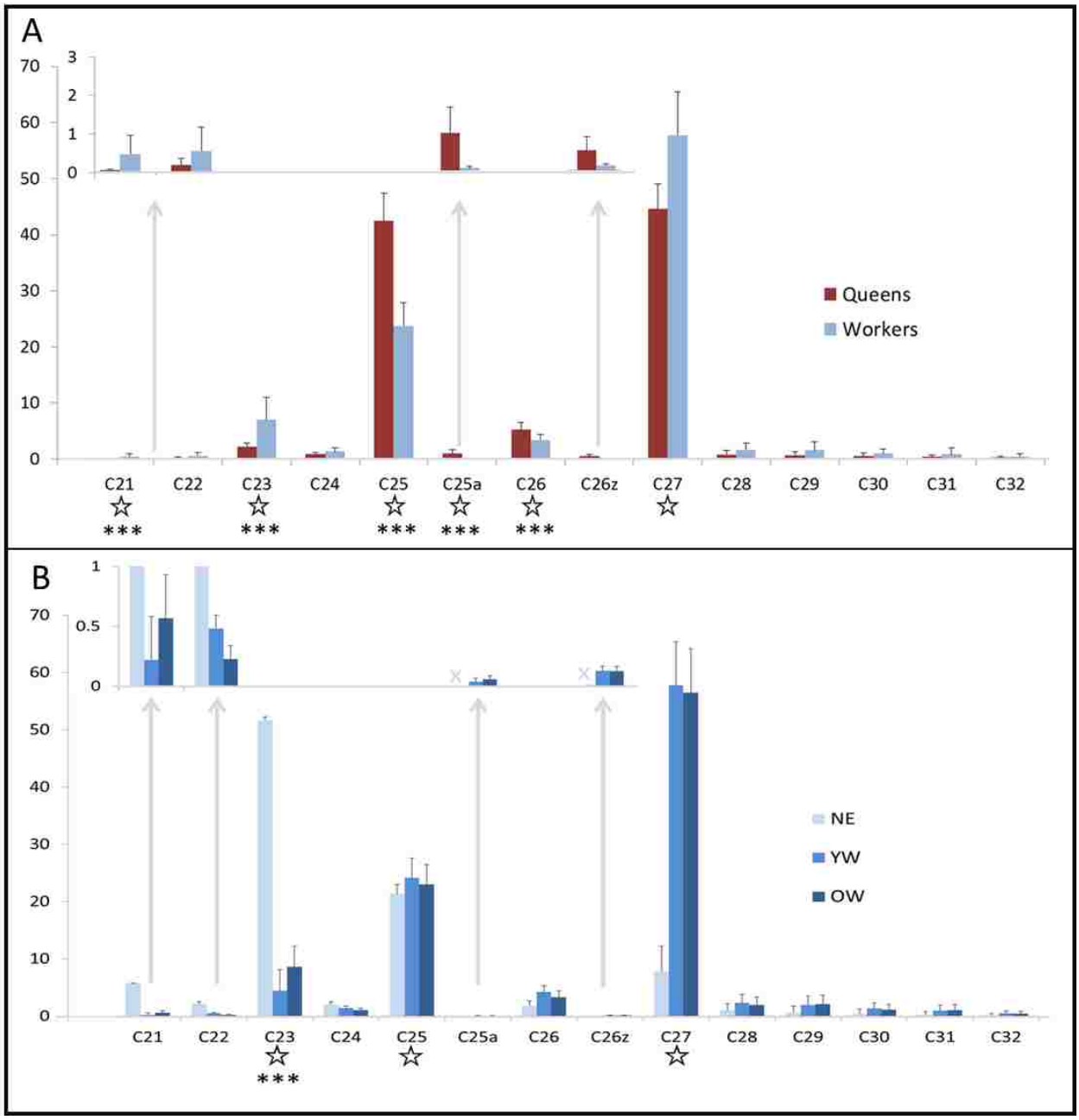
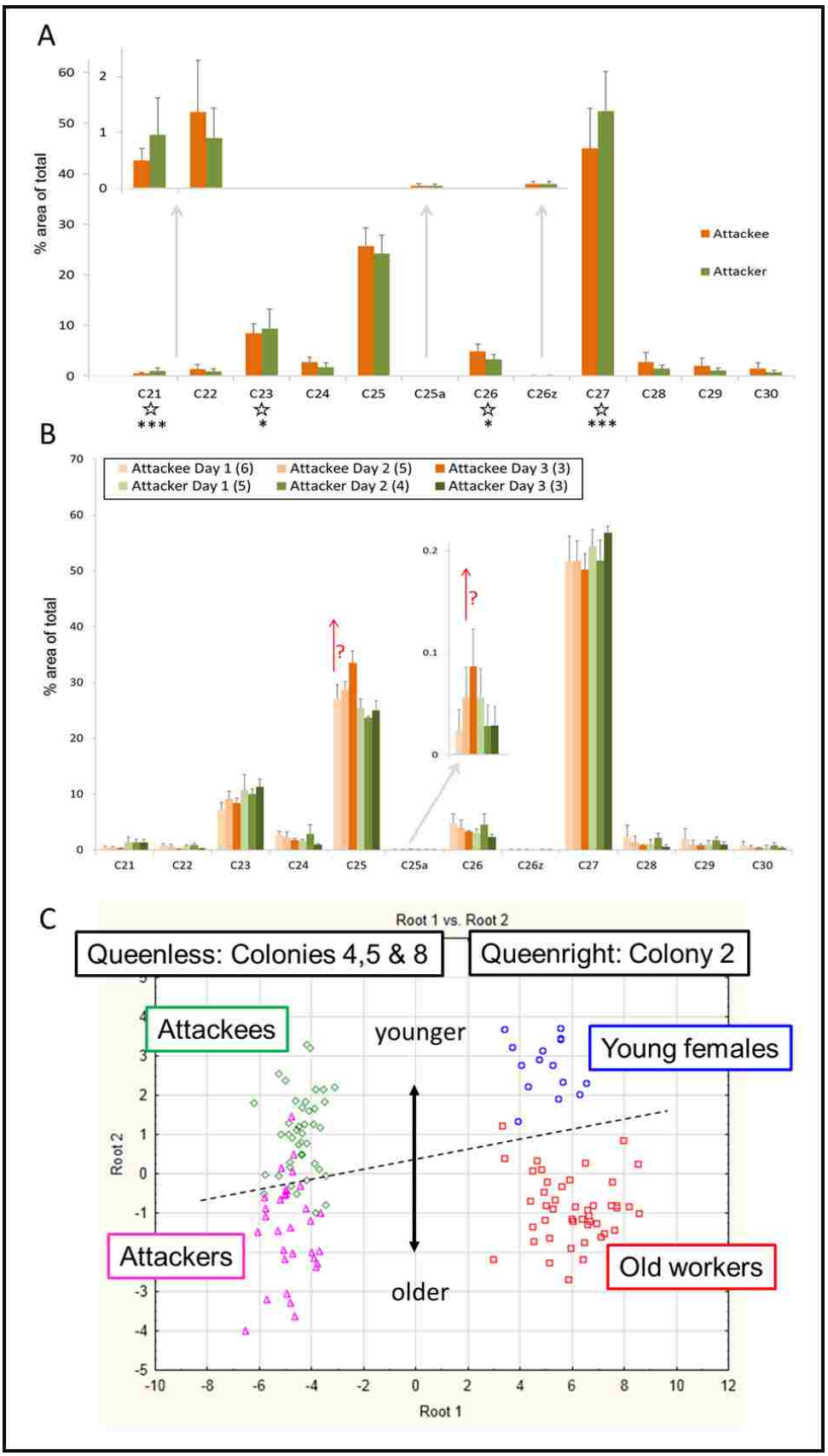


Figure 12. Cuticular hydrocarbon profile between co-existing types of females (mean % ± SD). Stars indicate the main contributors of separation, and * indicate a significant difference (***P<0.0001) in compound levels (log transformed). (A) Queens vs. workers from all Colonies 2-8. (B) Young wasps vs. very old wasps from Colony 2. Profile of four newly emerged (NE, callow) females is shown for comparison but was not included in the statistical analysis. “x” indicates compound was not detected.


A
% area of total
Attackee
Attacker
C21
C22
C23
C24
C25
C25a
C26
C26z
C27
C28
C29
C30
B
Attackee Day 1 (6)
Attackee Day 2 (5)
Attackee Day 3 (3)
Attacker Day 1 (5)
Attacker Day 2 (4)
Attacker Day 3 (3)
% area of total
C
Root 1 vs. Root 2
Queenless: Colonies 4,5 & 8
Queenright: Colony 2
Attackees
younger
Young females
Attackers
older
Old workers
Root 2
Root 1

(Previous page) Figure 13. Cuticular hydrocarbon profile between co-existing types of females (mean % ± SD). Stars indicate the main contributors of separation, and * indicate a significant difference (***P<0.0001; *P<0.5) in compound levels (log transformed). (A) Attackees vs. attackers from Colonies 4, 5 & 8. (B) Attackees vs. attackers over the first three days following queen removal from Colony 4. Red arrows suggest queen-associated compounds are rising (in proportion to other compounds) in only the attackees. Sample sizes for each group are given in parentheses. (C) Discriminant analysis of attackees and attackers from Colonies 4, 5 and 8, and young females and old workers from Colony 2. Attackees differ from attackers in the same direction as young females differ from old workers.

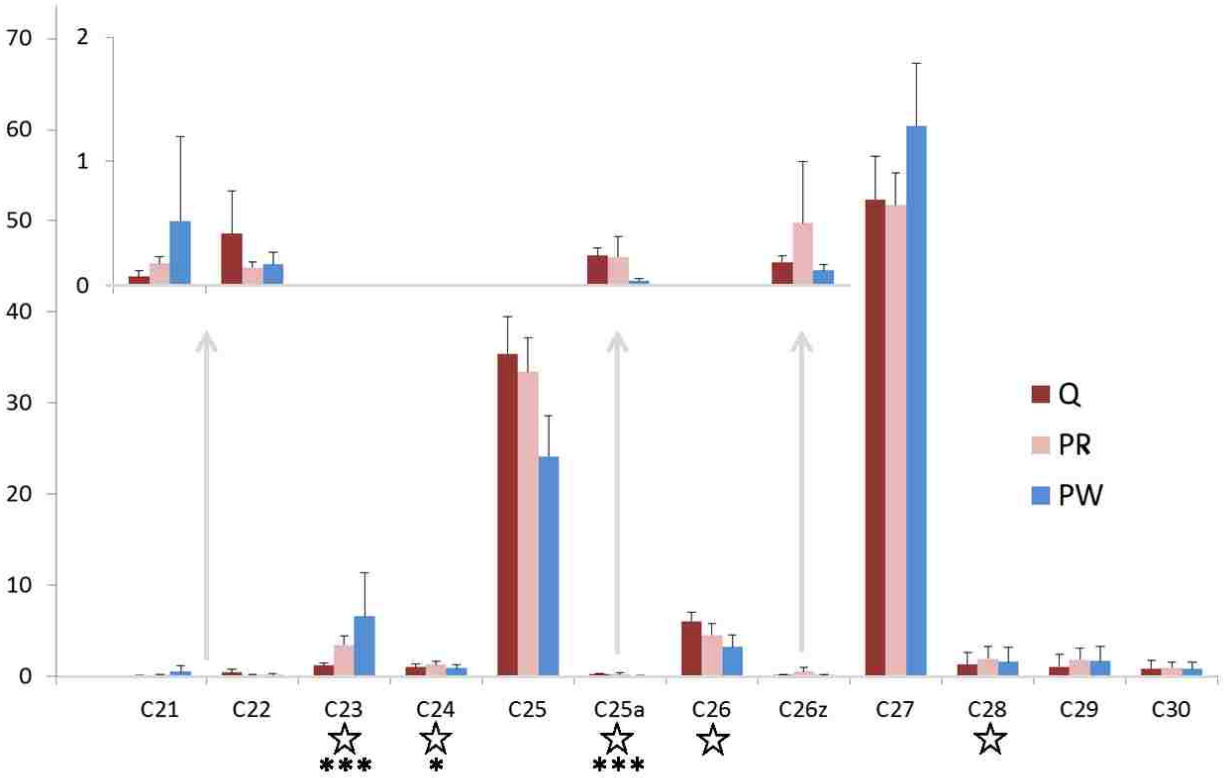


Figure 14. Cuticular hydrocarbon profile between co-existing types of females (mean % ± SD) from Colonies 4 and 5. Stars indicate the main contributors of separation, and * indicate a significant difference (***P<0.0001; *P<0.5) in compound levels (log transformed). PR: prospective reproductives; PW: putative workers; Q: Queens. Data from the queens were not included in the statistical analysis.

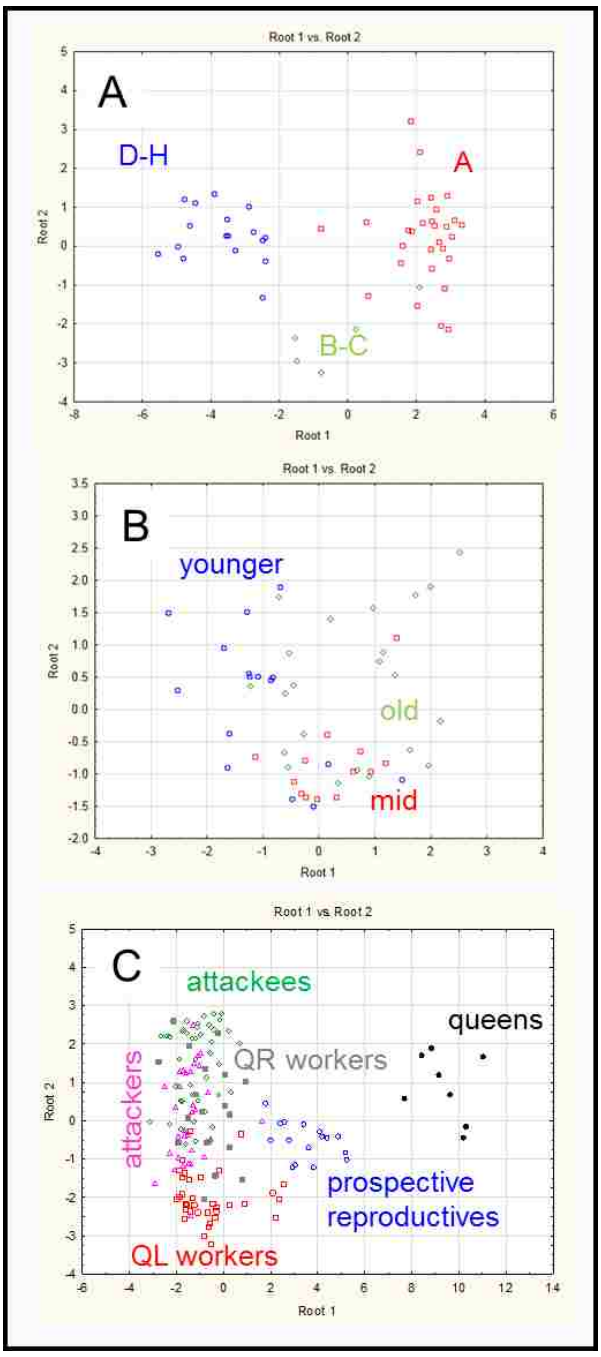


Figure 15. Discriminant analysis of samples from Colony 4 & 5 based on (A) ovarian status (see Fig. 2) (classification matrix=96%) and (B) relative cuticular age (young=2.5-3; mid=3.5-4; old=4.5-5) (classification matrix=67%). (C) Discriminant analysis of all queens, queenright (QR) workers, attackees, attackers, prospective reproductives and queenless (QL) workers from Colonies 4, 5 & 8.

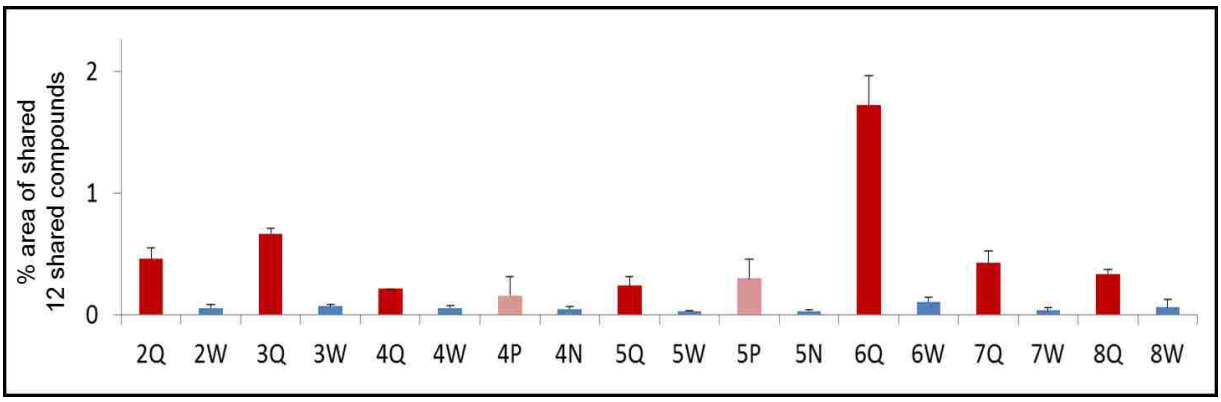


Figure 16. In all colonies examined, C25a (3methyl C25) was higher in queens (Q) than workers (W). C25a was also higher in potential prospective reproductives (P) than putative workers (N) removed at the same time. Number indicates colony. (mean%±SD).

Chapter 4:
The endocrinology of *Synoeca surinama*

Table of Contents

Introduction

The central objective for this chapter is to determine the underlying endocrine mechanisms regulating caste determination and functions in a caste monomorphic species of wasp for all the reasons stated in Chapter 3. As in *Polybia micans*, the astelocyttarus epinonine wasp *Synoeca surinama* separate out queens and workers in the adult stage and it is typically the youngest females on the nest who fill the void (West-Eberhard, 1977, 1981). As in other astelocyttarus wasps, such as *Metapolybia aztecoides*, even callow soft-bodied females, the most vulnerable individuals on the nest, are able to signal dominance upon emergence on a nest lacking queens (West-Eberhard, 1978, 1981). The workers, who ultimately decide who will reproduce and who will work, give these young females a free pass to join the next cohort of future reproductives. In this sense, *S. surinama* differs from *P. micans* since queen selection in the latter involved physical tests (Chapter 3). From a practical methodological perspective, the study of *S. surinama* promises more immediate answers given the architecture of their nest which lends itself to observational studies (Fig. 1).

In primitively eusocial wasp societies, where a single queen dominates the colony by force (e.g., most *Polistes* sp.), JH is important for sustaining reproductive growth and dominance-related aggression (Roseler, 1985; Roseler et al., 1980, 1984; Tibbetts et al., 2011a), especially in periods of social instability (e.g., queen succession) (Tibbetts and Huang, 2010). In contrast, JH appears not to be important for maintaining ovarian growth in oligarchic societies of an advanced swarm founding wasp, *Polybia micans* (Chapter 3). Instead, in *P. micans*, JH appears to be important for priming the ovaries of young prospective reproductives for future growth (when the queens are removed), and may influence dominance behavior and/or augment ovarian development in lone queens. In view of the fact that *S. surinama* and *P. micans* are Epiponini swarm founding wasps which postpone caste determination until the adult stage, a similar endocrine pattern could be expected for *S. surinama*. Yet phylogenetic analyses

conclude that the astelocyttarus clade is less derived than *Polybia* (Noll and Wenzel, 2008; Pickett and Carpenter, 2010), raising the possibility that JH has a more *Polistes*-like endocrine profile in *S. surinama*.

Methods

Wasps

All behavioral and physiological data were collected from *Synoeca surinama* [Hymenoptera: Vespidae: Epiponini].

Field studies

Field work was carried out on the campus of Universidade de Federal Sergipe (UFS), São Cristóvão, Sergipe, Brazil. The paper cells of a *Synoeca* nest, like those of the closely related *Metapolybia*, are built directly onto the substrate, usually a tree trunk or thick branch. As the construction and elongation of cells are underway, workers begin constructing walls on either side of the incipient comb. The walls eventually arc over the cells and meet to complete the envelope (Fig. 1). The initial nest resembles the back of an armadillo, hence the Brazilian name for its appearance (“tatu”), with a small turret-like passage pointing upward. When a nest expands, it expands upward, creating obvious compartments with every extension event (Fig. 1C).

The nest architecture of *Synoeca* and closely related genera has provided field biologists the opportunity to observe all activity inside a colony due to the lack of tiered combs (West-Eberhard, 1978). In my experience, established nests with older larvae rarely swarm in response to envelope removal. An immediate aggressive response is typical, but workers acclimate to the disturbance quickly, begin the rebuilding process, and do not seem to notice the observer thereafter. To avoid stress-related endocrine responses, females were not

sacrificed within the first 2 days of envelope removal. The paper material of a *S. surinama* nest is brittle, and so once a section of envelope is removed, it cannot be easily reinstated (cf. nest envelopes of *Metapolybia* which can be folded back, as if on a hinge, and shut when the observation session concludes) (West-Eberhard, 1978). The destruction of the nest envelope unavoidably induces a drastic change in task allocation toward restoring the envelope (e.g., building and pulp foraging). Thus, when aspects of worker behavior (e.g., temporal polyethism) were investigated, only small pieces of envelope were removed so as not to overwhelm the worker force with building demands. All nests of *S. surinama* were studied *in situ*, anywhere from 1 to 12 meters off the ground.

Wasps were color marked with oil-based Sharpie pens according to status or task performed (queens, builders, forager of pulp, caterpillar meat, nectar and water). To limit the effect of possible circadian endocrine changes (Zera, 2007), the vast majority of wasps were collected for processing from 13-17h. Wasps were placed in clean glass vials and buried in ice (0ºC) within 15 seconds of removal. Collection events for individual wasps almost always included multiple types of females, with the relevant pair determined by the objective of the assay (e.g., queens vs. workers).

Laboratory studies

Procedure for collecting haemolymph, cuticular hydrocarbons and ovary measurements

Wasps were transferred on ice to the lab for processing. After 20-120 minutes of 0ºC, the anesthetized wasp was removed and immobilized against a substrate with intersecting insect pins under a stereomicroscope. Since cold anesthesia for a period exceeding 2 hours is known to significantly affect juvenile hormone titers in honeybees (Lin et al., 2004), all wasps were bled within this time frame unless otherwise noted. Using a 5 µl graduated microcapillary (Drummond Scientific Company) pulled to a point over a flame, 2 to 10 µl of hemolymph was withdrawn from

between the anterior-most segments of the gaster. Samples destined for JH measurement by radioimmunoassay (RIA) were transferred to 500 µl of acetonitrile inside a 2 ml screw-top glass vial capped with a Teflon-lined rubber septum. Samples destined for ecdysteroid measurement were preserved in 500 µl of methanol. Subsequent to bleeding, cuticular hydrocarbons (CHCs) were extracted from females by placing them in 2 ml of hexane for 2-2.2 minutes. The ovaries were carefully removed in cold E&B Ringer's solution (7.5g NaCl and 0.35g KCl / 1L distilled water (Ephrussi and Beadle, 1936)), photographed with a Leica EZ4D Microscope Camera and were measured using Leica Application Suite software (Leica Microsystems). Ovaries were then placed in 500 µl of methanol for ecdysteroid measurement. All hormone and CHC samples were kept at -20ºC until transport to the Universidade de São Paulo, Ribeirão Preto, where they were processed.

Juvenile hormone sample preparation (extraction)

JH was extracted from the haemolymph following a liquid-phase separation protocol developed for honeybees (Huang et al., 1994). The acetonitrile extract was combined with 1 ml 0.9% NaCl and 1 ml hexane in a 5 ml glass tube. After a thorough vortexing, the phases were allowed to separate on ice for 10 minutes, followed by centrifugation at 700 *g*. The supernatant hexane phase, containing the JH, was transferred to a new tube and the hexane extraction was repeated twice. The pooled hexane phases were dried by vacuum centrifugation and the extraction residues were redissolved in 50 µl toluene (containing 0.5% propanediol) and transferred to RIA glass vials. Just prior to the RIA, the solvent was removed by vacuum centrifugation.

Juvenile hormone titer analysis by radioimmunoassay

The RIA was performed according to the protocol devised by Goodman *et al*. (Goodman et al., 1990). In preparation, the JH-specific antiserum was diluted 1:1250 in phosphate buffer containing 0.1% bovine serum albumin and 0.1% rabbit immunoglobulin G. As a tracer, we used

[10-^{3}H(N)]-JH III (spec. activity 19.4 Ci/nmol, Perkin Elmer Life Sciences, Waltham, MA, USA), diluted in 0.1 M phosphate buffer (pH 7.2-7.4) (made from 0.2 M mono- and dibasic potassium phosphate stock solutions and 0.02% sodium azide) to 6000-6500 cpm/50 ml. Synthetic JH III (Fluka, Munich, Germany), the lone species of JH known in Hymenoptera (Goodman and Cusson, 2011), was used as a non-radioactive ligand. Two standard curve replicates were set up to cover a range of 25 pg – 5 ng.

After an overnight incubation at 4ºC, saturated ammonium sulfate was added (50% final concentration) to precipitate the antibody-bound JH-III. Standard curve values were log/logit transformed, and a linear regression was produced to determine JH-III equivalents (pg/ml hemolymph) (i.e., titers) for each sample.

Ecdysteroid sample preparation

Haemolymph samples in 500 µl in methanol were cold centrifuged (4^{o}C) for 10 minutes to pellet the protein precipitate and impurities. The supernatant was directly transferred the RIA glass vials and dried by vacuum centrifugation.

To measure the ecdysteroid content of queen ovaries, which are particularly rich in lipids (which interfere with ligand-binding and the precipitation of the antigen-antibody complex), an additional purification step is needed. After centrifugation and decanting an aliquot or the entire ecdysteroid sample, the methanol was evaporated by vacuum centrifugation and then the pellet was resuspended in 1 ml 30% methanol. The sample was then slowly loaded into SepPak-C18 cartridge (Waters, WAT051910) with a 1 ml disposable syringe. After twice passing 1 ml 30% methanol through the cartridge, the eluate (containing polar ecdysteroid conjugates) was discarded. The cartridge, containing the free ecdysteroids, was eluted by two consecutive rinses with 60% methanol, and the pooled eluates were then dried by vacuum centrifugation and redissolved in 100% methanol (Geva et al., 2005).

Ecdysteroid analysis by radioimmunoassay

Ecdysteroids were quantified by RIA, as previously described (Feldlaufer and Hartfelder, 1987), using an antiserum prepared against a hemisuccinate derivative of ecdysone (Bollenbacher et al., 1983; Warren and Gilbert, 1986). In preparation for the RIA, the 20E-specific antiserum was diluted 1:1300 in 0.1 M phosphate buffer (pH 7.2-7.4) (made from 0.2 M mono- and dibasic potassium phosphate stock solutions and 0.02% sodium azide) containing 0.1% bovine serum albumin and 0.1% rabbit immunoglobulin G. As a tracer, we used [23,24-^{3}H(N)]ecdysone (Perkin Elmer) (NEN, spec. act. 102 Ci/mmol), diluted in phosphate buffer (see above) to 5000-6000 cpm/100 ml. Standard curves were established using a synthetic 20-hydroxyecdysone (20E; Sigma) as a nonradioactive ligand. Accordingly, results are expressed as 20E equivalents (pg). Two standard curve replicates were set up to cover a range of 25 pg-2 ng.

After an overnight incubation at 4ºC, saturated ammonium sulfate was added (50% final concentration) to precipitate the antibody-bound 20E. Standard curve values were log/logit transformed, and a linear regression was produced to determine 20E equivalents for ovarian content or titers for each sample.

Exogenous JH application

Females were treated with either JH III (Sigma), the only known type of JH in Hymenoptera, or methoprene, a JH mimic (Zoecon). For Experiments 1 and 2, 10 µg of JH III was dissolved in 1 µl cyclohexane and topically applied to the gaster of anesthetized wasps on three consecutive days. For Experiment 3, 10 µg of methoprene in 1 µl cyclohexane was applied to wasps at the adult age of 3 and 6 days. As a control for all experiments, 1 µl of cyclohexane was used. Some females in Experiment 3 were anesthetized but were not treated. All wasps were kept in isolation for 45-60 minutes before they were introduced back onto the nest to reduce the spread of topically applied JH and methoprene to nest mates.

Cuticular hydrocarbon analysis

The epicuticular surface hydrocarbons were extracted in 2 ml hexane for 2-2.2 minutes. After the solvent was evaporated under a fume-hood, the apolar extract was suspended in 50 µl of hexane. One µl of this was injected into a combined gas chromatography-mass spectrometer (Shimadzu, model QP2010). Separation was achieved on a DB-5MS column of 30 m, with a helium gas carrier at 1.0 ml min^{-1}. For most samples, the oven temperature was initially set to 150º C, and ramped up 3º C min^{-1} until it reached 280º C and held for 20 minutes. Analyses were performed in the splitless mode. The mass spectra were obtained by 70 eV ionization. The chromatographs were analyzed with GCMS solutions (Shimadzu).

Statistical analyses

Hormone data

Although many hormone samples were collected, sample size for certain statuses were often very low (e.g., newly eclosed females), precluding tests of significance. From nest to nest, consistent patterns of hormone titers were observed between types of females, but the range of JH titers for a given nest was not always the same. This is likely due to the fact that these colonies were studied in the field and are subjected to all kinds of environmental influences, the most obvious being climate. Also, many of the RIAs were carried out independent of one another over a two year period. Therefore, I looked for differences in pooled data using the Least Squared Means Tukey Method test which allowed me to use the following factors, when necessary: 1) status, 2) Colony origin and 3) Colony condition (queenright vs. queenless). In other cases, a *t*-test or ANOVA sufficed. For extremely non-normal data, Kuskal-Wallis rank sums test was used, and in the case of multiple comparisons, the Steel-Dwass method for all pairs was employed. All hormone and ovarian data were analyzed using JMP 10.0.

Cuticular hydrocarbon data

Principal components analysis (PCA) was used to define the main components to be compared. Compounds missing in most individuals of an analyzed group as well as compounds contributing less than 5% to the first two factors, as indicated by the PCA, were excluded from the statistical analysis. The relative concentrations of the compounds used in the discriminant analysis were readjusted to 100%. Following this, a stepwise discriminant function analysis was used to observe if combinations of variables could be useful in the predicting group. Wilks' λ values were used to verify the individual contribution of each variable to the model.

To avoid errors in the compositional sample data, the area under each peak was transformed according to the following formula: $Z = \ln[Ap/g(Ap)]$, where Ap is the area under the peak, $g(Ap)$ is the geometric mean for each individual compound group and Z is the transformed peak area (Aitchison, 1986). I compared proportions of compounds for each wasp group using the *t*-test. The statistical analyses were performed using the software Statistica 10.0.

Determination of ovarian status

Queen ovaries were quantified by counting the number of oocytes > 75% the length of a normal egg. Non-queen oocytes were measured using ImageJ (NIH) and oocyte length is given as a percentage of a fully grown egg.

Determination of relative age

Relative age was estimated by scoring (blind) the degree of apodeme darkening on the 5th abdominal sternite (West-Eberhard, 1969). Pictorial representations of this feature and the scores assigned are shown Fig. 2.

Results

Colony demographics

In total, 12 colonies were studied (Table 1). Queen number ranged from 1-35 while worker number ranged from ~65-300. All nests with exception of Colony 6 had produced at least one generation of females, and males were found only in Colony 4.

Colony	Nest state	Queen number	Oocytes > 75% egg	Worker #	Nest compartments	Males	Month
1	post-emergence	?	not sacrificed	~200	2	?	Feb
2	post-emergence	?	not sacrificed	~300	3	?	March
4	post-emergence	4	7.8 (4-11)	~65	1	yes	July
5	post-emergence	~35	4.4 (2-7)	~200	2	no	Sept-Nov
6	pre-emergence	12	6.3 (4-7)	~200	1	no	May
7	post-emergence	7	5.3 (3-9)	~100	1	no	June
8	post-emergence	11	5.8 (4-7)	~150	2	no	Oct
9	post-emergence	1	12	~70	1	no	Nov
10	post-emergence	5	13.5 (10-18)	~250	1	no	Nov
A	post-emergence	1	not sacrificed	~100	1	no	Nov
B	post-emergence	5	not sacrificed	~200	3	?	Nov

Table 1. Basic information for colonies used in this report. Nest state indicates whether the nest had produced the first generation of adults. Worker number was estimated. Nest compartments indicate the number of nest expansion events (e.g., Fig. 1A has 3 compartments and Fig. 1B has only 1).

Behavior

Queens

In agreement with previous reports (West-Eberhard, 1977), queens and workers of *S. surinama* engage in ritualized acts of aggression: workers perform the queen dance, and queens bend toward dancing workers and to one another (Fig. 1D), often in static aggregations inside the nest but off the comb. Non-queens were never seen to lay an egg, but on two occasions a fully

established queen was observed to add pulp to the nest envelope (i.e., build). In each colony, queens were of the same 'cuticle' age, indicating that they had become queens together.

Queen succession

When all queens of a nest were removed, the next cohort of potential queens (i.e., abdomen benders with developing ovaries) was typically comprised of the youngest females present, including females who eclosed within a few days *after* queen removal (i.e. they were still pupae when the queens were removed). In fact, the bender cohorts from Colonies 7 and 8 consisted entirely of females who emerged after queen elimination, despite the presence of 1-8 day old females in Colony 7 when the nest was made queenless (only workers > 3 weeks of age were present on Colony 8; see JH Application Experiment I). The benders from Colony 5, 9 and 10 included both young adults present on the nest at the time of queen elimination and those who emerged afterward. In a nest which was not producing new adults, a subset of young workers began bending (Colony 4). Colony 6, a pre-emergence nest, lacked pupae and workers < 4 weeks. Despite their age and ovarian condition (all possessed borderline filamentous ovaries), over 20 of these workers initiated bending, and those sacrificed a week later had well developed ovaries. Thus, most if not all workers of *S. surinama* are totipotent, although as previously reported for *M. aztecoides* (West-Eberhard, 1977), it is typically the youngest females on the nest who become the next queens.

In Colony 6, females who switched from working to bending (n=20) varied in 1) bending intensity (angle and frequency), 2) location on the nest (on or off the comb) and 3) outright aggression (e.g., attacking other benders). These behavioral characteristics did not always correlate, but those who displayed in the middle of the comb tended to be extreme benders, and those who were at the corners of the nest tended to bend only slightly. This variability in bender 'personality' was absent in colonies where young females were present, who generally assumed bending within an hour of emergence. Females who emerged after the cohort of queens had

been set, however, were repeatedly attacked and relegated to worker status. The aggressive interactions, as has been described in honeybees, could be characterized as 'mauling' (Sakagami, 1954) or 'mandibulation' (Visscher and Dukas, 1995) of one wasp by another, and sometimes involved ritualized stinging sequences. Indeed, the interaction closely resembled aggressive gestures performed by queens toward lower ranked females in hierarchical based societies of *Polistes*. These behaviors are thought to lead to ovarian regression in those who receive them (Reeve, 1991).

Temporal polyethism

Age-related changes in behavior, or temporal polyethism, was evident in colonies where young females were observed. Young females, who remained idle for the first few days after eclosion, often formed clusters away from the queens, began building anywhere from 2-4 days after emergence and were almost never seen to forage before the age of 10 days. The older workers foraged and sometimes built. Specialized foragers of materials (e.g., nectar, water, pulp, meat) were common but I did not study these wasps in detail.

Ovarian development in young females from queenright and queenless colonies

Oocyte length (% of fully grown egg) was assessed in young queenright and queenless females from Colonies 5 (~35 queens) and 7 (7 queens). Females eclosed with partially developed ovaries. Young queenright females experienced limited oocyte growth before their oocytes begin to regress after a few days, becoming completely regressed and filamentous as early as 7 days after eclosion (Fig. 3).

The ovaries of queenless (i.e., queen-destined) females and of queenright (i.e., worker-destined) females began to show differences at day 3 and grew more disparate with time (Fig. 3).

JH titers

Queens vs. workers

Queens consistently show higher JH titers than did workers (left side of Figure 4A-G; blue x-axis for each graph indicates queenright conditions). In a pooled analysis including data from all queenright colony conditions, queens (n=50) had significantly higher JH titers than workers (n=129) and newly eclosed females (n=12) (Least Squared Means Tukey HSD test; factors: status and colony) (Fig. 5A). The age and status of queenright workers who were sacrificed varied from nest to nest: Colony 4 consisted of builders and foragers (cuticle age: 2-3); Colony 5 consisted only of 1-3 day old builders or worker-destined females; Colony 6 was comprised of only middle to old workers (cuticle scores 3.5-5); Colony 7, 8 & 9 consisted of workers from all ages; and Colony 10 was comprised of young and middle aged builders and foragers (ranging from 1-3.5 cuticle age). Queenright newly eclosed females, when present, had baseline titers within the range of their worker nest mates (Fig. 4C, E-G). Thus, on a nest with its envelope removed, JH is relatively high in displaying reproductives but low or undetectable in a variety of workers and newly emerged females.

Queens vs. queens

In Colony 5, oviposition events were recorded over an 8 day period (10h/day) in order to rank queens according to a direct measure of reproductive dominance. Most females were directly observed to oviposit 3-5 times (n=24). Those who oviposited the least (0-2 eggs; n=6) or most (6-8 eggs; n=5) were bled for JH measurement. There was no difference in JH titer between queens with low and high fecundity (n=11) (Fig. 4E). Also, on this colony, a queen was continuously attacked by workers and was likely in the process of being demoted or expelled from the nest. Both she and a reference queen had JH titers within range of the queens sacrificed above (data not shown). These data suggest that JH is not correlated with reproductive status or behavioral dominance among queens.

Queenless benders vs. others

When a colony was made queenless (red axes of Fig. 4), certain females began bending. This behavior signaled to their nest mates that they intended to become a member of the next generation of queens. These benders, sacrificed from 1-2 days after queen elimination, consistently had higher JH titers than workers who continued to work in the absence of queens. As mentioned above, some benders eclosed as future queens while others switched from working to bending. In a pooled analysis including data from all colonies in a queenless state, workers-turned-benders (n=36) had higher JH titers than benders (n=19) who had eclosed on a queenless nest, workers (n=54) and newly eclosed wasps (n=7) (Fig. 5B) (Least Squared Means Tukey HSD test; factors: status and colony origin). JH titers did not correlate with any of the three 'personality' traits described above for Colony 6. Finally, newly emerged females who assumed bending had low JH titers (Fig. 4F).

In Colony 6 and 7, some benders were allowed to develop their ovaries over the next week (Fig. 4C-D; green x-axis). In Colony 6, the JH titers of the established, accepted benders were extremely variable. One bender was being chased and attacked, and a former bender was observed working. Both of these females, which I refer to as 'defeated benders', had baseline JH titers. In Colony 7, the 4-8 day old females had been accepted as future queens (i.e., established benders). Those younger than these established benders (1-4 days old), who eclosed after the cohort of queens had been established, frequently huddled instead of working and were often attacked. These suppressed 'huddlers' had very low JH titers. In a pooled analysis including data from both of these colonies, the established benders (n=11) had higher JH titers than the huddlers (n=10) and their worker (n=16) counterparts (Fig. 5C) (Least Squared Means Tukey HSD test; factors: status and colony origin). Probably on account of the small sample size, newly eclosed females did not significantly differ from any group. Huddlers were also observed in Colony 4: they were not pooled in the above analysis, were not among

the youngest females on the nest (i.e. had cuticle scores of 3 and 3.5) and had filamentous ovaries and low JH titers (Fig. 4B).

In a grand analysis including females from queenright and queenless conditions, queens (n=50) and benders (n=66) (encompassing both work-turned-benders and females who bent upon emergence) had higher JH titers than workers (n=205) and newly emerged females (n=23) (Fig. 5D) (Least Squared Means Tukey HSD test; factors: status, colony origin and colony condition (QR/QL)).

Queenright benders vs. queenless benders

In Colony 10, removal of 3 of 4 queens (single queen reign is indicated by the light blue x-axis bar in Fig. 4G) led to the emergence of benders in queenright conditions, the only time this was observed. All newly emerged, idle females (1-2 days old) and a few young working females (3-5 days old) began bending. These females were chased and attacked by the lone queen and some of the remaining workers. Of these groups, only the queen had elevated JH titers (Fig. 4G). Once the last queen was removed, JH titers were clearly elevated in the three queenless benders on the following day (Fig. 4G, far right). Due to the low samples sizes, a non-parametric test (Steel-Dwass Method) was used to look for differences between these groups (Fig. 5E). Queens (n=4) and queenless benders (n=3) had significantly higher JH titers than pooled queenright + queenless workers (n=25) but not queenright benders (n=10). The latter statistical comparison is compromised by a small sample size.

Young queenright workers vs. young queenless benders

JH titers were also measured in young queenright and queenless females through the first 8-9 days of adulthood. First, I bled the same individuals twice 4 days apart, covering ages 0 to 8 days (e.g., some females were bled and released on day 2, then bled and sacrificed on day 6). Fig. 6A shows an apparent drop of JH titers with age, concomitant with ovarian regression (Fig.

3), but a significant difference (*t*-test) was also obtained for 4 day old females who were bled first (and released; 4') and bled second (having been bled on day 0 and sacrificed on day 4; 4") (Fig. 6A). This difference could reflect an endocrine-mediated response to injury, and so repeated bleeding experiments were not employed elsewhere in this study.

A month later, for the same colony, I compared JH titers of queenright and queenless females through the first 3 days of adulthood (Fig. 6B). In both conditions, newly emerged females (<24 hours) had low JH titers. In queenless females, who were also benders, a rise in JH was seen in 1 day old females and was sustained through day 3. In queenright females, JH titers remained low through the first 48 hours and rose slightly in day 2 and day 3 females. A significant difference is found between pooled 1-3 day old queenright vs. queenless females (*t*-test; P=0.009).

In Colony 7, 0-9 day old adult females in queenright and queenless conditions were compared (Fig. 6C). Overall, JH titers were much lower in this nest. Older (4-8 day old) queenless benders tended to have higher JH titers than queenright females (0-9 days) and the younger cohort of suppressed females (0-4 days).

JH and ovarian state

JH positively correlated with oocyte length (% of fully grown egg) among all nest mates (including both queenright and queenless conditions) in colonies 4 (n=35; R^2=0.41; ANOVA P<0.0001), 6 (n=80; R^2=0.28; P<0.0001), 7 (n=70; R^2=0.8; P<0.0001), 8 (only queenright conditions; n=22; R^2=0.83; P<0.0001), 9 (n=53; R^2=0.17; P<0.005) and 10 (n=43; R^2=0.57; P<0.0001) (Fig. 7). A positive correlation was not observed for colony 5 (P=0.13) due to the abundance of queenless females who had recently initiated bending (with new benders removed, P<0.0001). JH titers were always higher than expected (based on oocyte length) in new benders, and indeed, accepted benders of all kinds (i.e., future and actual reproductives)

within a given nest had elevated JH titers compared to non-benders, suppressed benders and huddlers (Fig. 7A,C,D,G).

JH and temporal polyethism

Builders and foragers were sacrificed on Colonies 1 and 2 where only a small piece of the envelope was removed (see Methods). There are a range of JH titers, with nectar foragers having the highest of all (Fig. 8). Such a range of titers was not typically observed in workers where most of the envelope was removed (Fig. 4).

Ecdysteroids

Ovarian ecdysteroids

Ecdysteroids were detected in all queens assayed, and ovarian ecdysteroids correlated with queen ovary size (number of oocytes > 70% length of egg) (n=36; R^2=0.346; ANOVA P=0.0002) (Fig. 9A). Queens from Colony 5, who were split based on fecundity (high vs. low), showed no difference in ovarian ecdysteroids (n=11), and 2 defeated queens contained ecdysteroids within the range of these queens. Among non-queens, ovarian ecdysteroids clearly correlate with oocyte length in pooled workers and benders from colony 4 (n=15; R^2=0.86; ANOVA P<0.0001) (Fig. 9B).

Ecdysteroids in the hemolymph

Circulating ecdysteroids were measured in queens and workers in two separate colonies. In Colony 4, ecdysteroids were only detected in 18 of 23 samples which included queens (n=4), benders (n=4) and workers (n=15). Of these samples, only four of the 15 workers registered positive titers, two of which had values above 85 pg/µl despite having filamentous ovaries. Given that the ecdysteroids are only known to be produced by the ovaries in adult Hymenoptera, it is likely these values are false positives. A separate RIA run for queens and

workers from Colony 8 produced detectable but low titer values (<7 pg/µl), and there was no difference between queens and workers (Fig 9C).

Effects of exogenous JH

Experiment 1: JH III application to queenless workers

On a nest containing brood of all ages, all queens and workers <3 weeks of age were removed (Colony 8). JH III and cyclohexane was applied to 46 workers (23 each) for the first three days following queen removal. There were no obvious differences in behavior between JH III-treated, cyclohexane -treated or non-treated wasps (e.g., no female initiated bending and no attacks were elicited from nest mates). The nest did not produce newly emerged females in the first two days of treatment, and callow females were removed when they appeared on the third and subsequent days (data not shown).

Experiment 2: JH III application to young prospective reproductives

After finding no effect of JH III treatment on old workers on Colony 8, I redirected treatment toward newly emerged, queenless females (one treatment per day for the first 3 days after eclosion). The first 6 females to eclose after queen removal assumed bending and were not treated. The following 15 females to emerge joined the cohort of future queens, irrespective of treatment (JH III: n=7; cyclohexane: n=8). Females who emerged 8-9 days after queen removal (JH III: n=2; cyclohexane: n=3) engaged in bending behavior but were attacked (i.e., mandibulated) and eventually became workers. The following 22 females were also attacked (JH III: n=11; cyclohexane: n=11), were never observed to bend and all became workers (Fig. 10). Thus, JH III treatment had no effect on a young female's ability to enter the legion of queens or initiate bending.

Experiment 3: Methoprene application to young queenright workers

To determine the effect of exogenous JH on ovarian development, I treated workers from two queenright colonies (Colony A and B) on day 3 and 6 of adulthood with the JH mimic methoprene or solvent (cyclohexane). Colony A, a monarchic nest, also included non-treated wasps who were anesthetized alongside the treated females. Colony B was oligarchic as it had 6 queens. By day 4 on Colony A, methoprene-treated females were receiving attacks from fellow workers. By age 8, the attacks were unremitting, and methoprene-treated females were rarely seen, eventually being evicted from the nest by age 9 or 10 days. Ovarian measurements and cuticular hydrocarbon extractions (see below) were taken on day 7. Young workers from Colony A and B were monitored and treated at the same time. Observations were restricted to Colony A although attacks were also observed in Colony B. In both nests, only methoprene-treated wasps were observed to be attacked.

The attacks, performed by older workers, were suppressive in nature in that the aggressor would attempt to initiate trophallaxis with the methoprene-treated wasp. Often this interaction transitioned to gentle mandibulation of the methoprene-treated wasp who assumed a submissive posture (i.e., crouching) while enduring the aggravation. This type of behavior was seen in natural nests, and indeed, was observed in very young females of Colony A when the envelope was first cut open.

For this experiment, all six primary oocytes were measured to provide an in-depth description of the state of maturation of the ovary (Fig. 11). When the length of each primary oocyte length is counted as a sample (i.e., each ovary contributes 6 samples if accurate oocyte measurements are possible), methoprene-treated females had significantly longer oocytes than cyclohexane - and non-treated controls for Colony A (methoprene (n=120) vs. cyclohexane. (n=101): $P<0.0002$; methoprene vs. no treatment (n=34): $P=0.0033$; cyclohexane. vs. no treatment: no difference) (nonparametric, all pairs, Steel-Dwass Method) (Fig. 10A) and Colony B

(methoprene (n=90) vs. cyclohexane. (n=77): P=0.003) (nonparametric Kruskal-Wallis Test) (Fig. 11). When the mean primary oocyte length for each female is counted as a single sample, a significant difference was only observed between methoprene- (n=17) and cyclohexane - treated (n=20) females from Colony A (nonparametric, all pairs, Steel-Dwass Method), P-value of 0.05 in my book this is a significant P=0.05). When cyclohexane - and non-treated wasps are combined (i.e., the controls) and compared to methoprene-treated wasps, the difference is significant (P=0.05) (nonparametric Kruskal-Wallis Test). Moreover, in Colony A, 51% of oocytes of methoprene-treated females appeared to be viable (i.e., not irregular or in a state of obvious degeneration) compared to 35% of cyclohexane - and 27% of non-treated oocytes. In Colony B, there was almost no difference in the percentage of putatively viable oocytes (~35%), but 57% of oocytes from cyclohexane -treated wasps were significantly degraded compared to only 33% in methoprene-treated workers.

Cuticular hydrocarbons (CHCs)

Analysis of the worker cuticular waxes identified 22 unambiguous hydrocarbons (Table 2) which appeared in every sample at detectable amounts. Identified compounds were either alkenes or linear alkanes.

Table 2 (next page). Mean percentage of composition and standard deviation (SD) of cuticular hydrocarbons components in various types of females of *Syneoca surinama*. Colony(s) of origin noted on top. Gray rows indicate components which are specific to queens and princesses (i.e., prospective reproductives on a queenless nest). HC abbrev=hydrocarbon abbreviation; Ret. Time= Retention Time; dse=days since eclosion; QR=queenright; QL=queenless; D=days without queen.

				QR All Colonies				*All Colonies*		*manipulation: QR Colony A*						*manipulation: Colony B*			
				Queens		Workers > 4 dse		Callows (0 dse)		JHA-treated [7 dse]		Cyclo-treated [7 dse]		Non-treated [7 dse]		JHA-treated [7 dse]		Cyclo-treated [7 dse]	
Peak	**hydrocarbon / component**	**HC abrev.**	**Ret. Time**	**N=67**		**N=95**		**N=24**		**N=18**		**N=20**		**N=6**		**N=14**		**N=13**	
				mean	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD
1	Heneicosane	**C21**	17.18	**0.08**	0.04	**0.16**	0.44	**3.29**	0.89	**0.18**	0.08	**0.14**	0.09	**0.15**	0.11	**0.23**	0.06	**0.20**	0.09
2	Docosane	**C22**	19.79	**0.06**	0.09	**0.33**	0.47	**0.61**	0.20	**0.06**	0.02	**0.08**	0.08	**0.06**	0.03	**0.10**	0.02	**0.11**	0.04
3	Tricosane-alkene	**ZZ C23**	21.74	**0.04**	0.03	**0.24**	0.55	**2.33**	0.87	**0.62**	0.20	**0.27**	0.24	**0.30**	0.20	**0.68**	0.16	**0.41**	0.23
4	Tricosane	**C23**	22.52	**0.32**	0.17	**1.88**	2.13	**17.73**	5.54	**2.64**	0.67	**2.60**	1.44	**3.04**	1.62	**3.33**	0.41	**3.28**	1.16
5	Tetracosane	**C24**	25.08	**0.19**	0.22	**0.72**	1.09	**0.63**	0.28	**0.34**	0.07	**0.38**	0.11	**0.42**	0.13	**0.52**	0.07	**0.59**	0.13
6	Pentacosane-alkene I	**Z C25 II**	27.1	**16.92**	5.70	**5.95**	5.22	**30.57**	7.57	**23.34**	4.50	**12.97**	6.62	**11.48**	6.33	**16.39**	1.59	**10.52**	4.48
7	Pentacosane-alkene II	**Z C25 II**	27.196	**0.22**	0.11	**0.28**	0.14	**0.73**	0.20	**0.59**	0.10	**0.35**	0.14	**0.33**	0.14	**0.53**	0.08	**0.40**	0.17
8	Pentacosane	**C25**	27.87	**13.11**	3.40	**14.23**	6.05	**12.23**	3.10	**9.03**	2.68	**14.29**	3.27	**16.97**	4.68	**14.19**	4.10	**19.11**	3.88
9	Hexacosane	**C26**	30.1	**0.35**	0.26	**1.19**	0.76	**0.41**	0.33	**0.37**	0.14	**0.59**	0.15	**0.74**	0.14	**0.57**	0.08	**0.98**	0.23
10	Heptacosane-alkene (378)	**Z C27 (378)**	31.96	**2.87**	1.04	**4.35**	1.54	**4.76**	1.11	**6.15**	0.90	**4.62**	0.82	**3.28**	0.41	**5.15**	0.50	**4.16**	1.03
11	Heptacosane	**C27**	32.58	**4.21**	0.46	**7.19**	1.76	**1.25**	0.59	**4.66**	1.23	**7.58**	1.62	**8.06**	1.90	**5.35**	0.98	**6.98**	1.61
12	Octacosane	**C28**	34.8	**0.33**	0.36	**1.12**	0.89	**0.43**	0.63	**0.21**	0.06	**0.37**	0.09	**0.44**	0.15	**0.29**	0.04	**0.45**	0.10
13	Nonacosane-alkene (406)	**Z C29 (406)**	36.57	**3.20**	1.08	**2.85**	0.94	**2.72**	0.62	**4.84**	0.68	**3.13**	0.48	**2.54**	0.55	**4.04**	0.40	**2.71**	0.75
14	Nonacosane	**C29**	37.17	**9.20**	1.43	**7.95**	1.68	**1.66**	0.88	**7.01**	1.30	**10.17**	1.82	**11.13**	2.19	**7.65**	1.39	**8.32**	1.23
15	Triacontane-alkene (420)	**Z C30 (420)**	38.73	**0.33**	0.09	**0.66**	0.23	**0.46**	0.11	**0.88**	0.13	**0.61**	0.13	**0.48**	0.14	**0.90**	0.17	**0.62**	0.22
16	Triacontane	**C30**	39.23	**0.86**	0.34	**2.08**	0.86	**0.49**	0.62	**0.74**	0.25	**1.25**	0.27	**1.42**	0.33	**1.13**	0.10	**1.66**	0.33
17	Hentriacontane-alkene (434)	**Z C31 (434)**	41.08	**23.97**	2.76	**16.55**	3.54	**14.14**	3.50	**20.33**	2.94	**13.96**	2.41	**11.63**	2.29	**16.75**	1.95	**11.01**	3.32
18	Hentriacontane	**C31**	41.54	**20.71**	6.05	**19.03**	5.92	**2.24**	1.00	**11.90**	2.59	**17.89**	2.91	**18.87**	3.22	**13.11**	1.23	**16.42**	2.79
19	Docotriacontane (448)	**Z C32 (448)**	42.95	**0.17**	0.05	**0.54**	0.21	**0.25**	0.08	**0.51**	0.07	**0.40**	0.08	**0.33**	0.08	**0.58**	0.11	**0.48**	0.18
20	Docotriacontane	**C32**	43.38	**0.51**	0.23	**1.68**	0.76	**0.30**	0.29	**0.47**	0.26	**0.81**	0.21	**0.92**	0.19	**0.87**	0.12	**1.43**	0.41
21	Tritriacontane-alkene (462)	**Z C33 (462)**	45.18	**0.76**	0.27	**5.13**	1.85	**2.23**	0.82	**2.75**	0.47	**2.89**	0.90	**2.46**	0.55	**3.25**	0.85	**3.50**	1.33
22	Tritriacontane	**C 33**	45.67	**1.60**	0.50	**5.91**	2.18	**0.55**	0.27	**2.37**	1.28	**4.66**	1.22	**4.93**	0.90	**4.38**	0.74	**6.66**	1.63

				QL Colony 6								*QL & QR Colony 7*				*Colony 8*			
				Worker-turn-Benders (D1)		Workers-stay-Workers (D1)		Worker-turn-Benders (D7)		Workers-stay-Workers (D7)		Benders [QL 4-9 dse]		Worker [QR 4-9 dse]		New Queens		QR Workers	
Peak	**hydrocarbon / component**	**HC abrev.**	**Ret. Time**	**N=18**		**N=9**		**N=5**		**N=4**		**N=7**		**N=16**		**N=19**		**N=12**	
				mean	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD	**mean**	SD
1	Heneicosane	**C21**	17.18	**0.02**	0.01	**0.03**	0.01	**0.01**	0.00	**0.02**	0.02	**0.28**	0.05	**0.15**	0.08	**0.25**	0.03	**0.0651**	0.0119
2	Docosane	**C22**	19.79	**0.05**	0.01	**0.05**	0.02	**0.03**	0.01	**0.02**	0.00	**0.12**	0.04	**0.10**	0.03	**0.08**	0.05	**0.1117**	0.0593
3	Tricosane-alkene	**ZZ C23**	21.74	**0.02**	0.01	**0.09**	0.13	**0.14**	0.05	**0.16**	0.24	**0.87**	0.12	**0.22**	0.16	**0.31**	0.07	**0.0994**	0.036
4	Tricosane	**C23**	22.52	**0.36**	0.18	**0.51**	0.35	**0.48**	0.10	**0.48**	0.31	**3.23**	0.52	**2.34**	1.06	**2.24**	0.26	**0.6127**	0.1148
5	Tetracosane	**C24**	25.08	**0.27**	0.11	**0.25**	0.12	**0.25**	0.02	**0.15**	0.03	**0.27**	0.05	**0.34**	0.08	**0.27**	0.05	**0.1758**	0.0602
6	Pentacosane-alkene I	**Z C25 II**	27.1	**2.19**	0.65	**3.37**	3.01	**11.36**	2.40	**4.85**	3.92	**24.84**	1.49	**7.99**	3.24	**24.11**	1.31	**3.3449**	0.7891
7	Pentacosane-alkene II	**Z C25 II**	27.196	**0.24**	0.10	**0.25**	0.08	**0.38**	0.08	**0.34**	0.12	**0.53**	0.05	**0.30**	0.08	**0.49**	0.07	**0.2787**	0.0962
8	Pentacosane	**C25**	27.87	**11.19**	3.14	**10.35**	3.05	**17.24**	1.58	**9.83**	1.33	**12.68**	2.04	**16.28**	2.52	**11.07**	2.17	**9.0597**	0.8593
9	Hexacosane	**C26**	30.1	**0.92**	0.33	**0.87**	0.41	**0.60**	0.10	**0.56**	0.10	**0.22**	0.05	**0.73**	0.08	**0.25**	0.05	**0.6461**	0.1349
10	Heptacosane-alkene (378)	**Z C27 (378)**	31.96	**4.80**	1.41	**4.81**	1.53	**7.51**	1.73	**6.75**	1.98	**5.86**	0.58	**4.24**	0.54	**4.21**	0.43	**3.645**	0.8507
11	Heptacosane	**C27**	32.58	**6.87**	1.27	**6.99**	1.34	**7.55**	1.41	**8.19**	2.59	**2.77**	0.43	**5.92**	0.79	**3.61**	0.32	**7.8531**	0.7251
12	Octacosane	**C28**	34.8	**0.96**	0.44	**0.81**	0.53	**0.30**	0.06	**0.40**	0.10	**0.16**	0.03	**0.40**	0.08	**0.18**	0.06	**0.4708**	0.0761
13	Nonacosane-alkene (406)	**Z C29 (406)**	36.57	**4.03**	0.81	**3.52**	1.42	**4.45**	0.91	**3.88**	0.99	**5.39**	0.78	**3.54**	0.64	**4.06**	0.46	**2.4594**	0.6768
14	Nonacosane	**C29**	37.17	**8.05**	1.53	**7.06**	0.98	**8.73**	1.89	**8.55**	2.71	**4.85**	0.59	**7.26**	0.77	**6.85**	0.61	**7.8734**	0.5953
15	Triacontane-alkene (420)	**Z C30 (420)**	38.73	**0.94**	0.25	**1.07**	0.59	**0.83**	0.23	**1.06**	0.52	**0.91**	0.10	**0.79**	0.12	**0.65**	0.09	**0.579**	0.1219
16	Triacontane	**C30**	39.23	**2.33**	0.48	**2.20**	0.50	**1.10**	0.24	**1.99**	0.45	**0.47**	0.09	**1.28**	0.23	**0.58**	0.07	**1.9017**	0.1885
17	Hentriacontane-alkene (434)	**Z C31 (434)**	41.08	**20.54**	3.10	**18.90**	3.94	**15.68**	3.06	**16.32**	3.80	**23.66**	2.74	**17.76**	1.69	**23.46**	1.85	**16.845**	2.0337
18	Hentriacontane	**C31**	41.54	**19.69**	3.27	**21.20**	6.60	**15.95**	3.56	**22.12**	6.48	**8.55**	1.27	**16.45**	2.56	**13.85**	1.39	**25.375**	2.2721
19	Docotriacontane (448)	**Z C32 (448)**	42.95	**0.96**	0.23	**0.95**	0.19	**0.48**	0.14	**0.73**	0.27	**0.48**	0.04	**0.65**	0.13	**0.37**	0.06	**0.7592**	0.1002
20	Docotriacontane	**C32**	43.38	**1.77**	0.43	**1.95**	0.72	**0.71**	0.16	**1.65**	0.43	**0.26**	0.07	**1.15**	0.25	**0.33**	0.09	**1.9917**	0.2418
21	Tritriacontane-alkene (462)	**Z C33 (462)**	45.18	**8.03**	1.66	**7.55**	0.58	**3.39**	0.79	**5.58**	1.09	**2.50**	0.20	**6.34**	1.05	**1.52**	0.24	**6.6865**	0.9446
22	Tritriacontane	**C 33**	45.67	**5.77**	1.31	**7.23**	2.68	**2.83**	0.74	**6.38**	1.64	**1.09**	0.36	**5.77**	1.15	**1.26**	0.22	**9.1668**	1.1532

Queens vs. Workers

Queens (n=67) and workers over the age of 4 days (n=95) were pooled from all colonies from which epicuticular compounds were extracted (Colonies 4-8). A representative chromatogram for each phenotype is shown in Fig. 12A-B. Adult females 4 days and younger were excluded in this analysis because younger females experience a dramatic, age-related change in their CHC profile (see below). A stepwise discriminatory analysis based on all 22 shared compounds significantly separated individuals according to their status (Global Wilks' λ = 0.01489; $F_{16,145}$=599.68; p<0.001) (Fig. 13A). A Principal Components Analysis (PCA) reduced the variable (i.e., CHC) number to 16, and the discriminatory analysis based on these compounds produced similarly robust results (Global Wilks' λ=0.03855; $F_{11,150}$=340.06;p<0.001). However, the PCA identified different main contributors than the discriminatory analysis performed on all compounds (Fig. 12A). Z C25 (an alkene) is the only compound significantly higher in queens (Fig. 12A) across every colony studied (e.g., Z C31, which was determined by both PCA and non-PCA discriminant analyses to be a main contributor, is not higher in queens in every colony). Workers tend to have a higher proportion of longer hydrocarbons (C32, Z C33, and C33).

Callows vs. older females

Predictably, callow females (< 24h as adults) produce a dramatically different CHC profile than older females (Fig. 13A-B). This is similarly evident in the chromatogram (Fig. 12C). A stepwise discriminatory analysis based all shared compounds between callow females (n=24) and queens+workers (n=162) significantly separated individuals according to their age (Global Wilks' λ=0.00148; $F_{34,334}$=245.96; P<0.001). The PCA reduced the variable (i.e., CHC) number to 17, and the discriminatory analysis performed on these compounds also separated the groups, although the difference was less marked (Global Wilks' λ= 0.15546; $F_{15,170}$=61.570; p<0.001). The PCA-based discriminatory analysis identified different compounds than the discriminatory

analysis performed on all compounds (Fig. 13A, bottom). A canonical plot of a discriminatory analysis performed for all queens, workers and callow females is shown as an inset in Figure 13B.

Young workers vs. young benders

The CHC profile of newly emerged females became more worker-like or queen-like, depending on the social context (Fig. 14). For all maturing females, smaller CHCs (e.g., C21, Z C23 and C23) decreased in proportion while larger CHCs (C29, Z C31, C31) increased. A similar trend is seen in worker-destined females which see a dramatic decrease in Z C25 and an increase in larger compounds such as C32, Z C32 and C33. Maturing benders show just the opposite pattern, and this marks the emergence of a queen-like CHC profile. In support of this, a discriminatory analysis for Colony 7, where both 4-9 day old queenright workers and queenless benders were sacrificed, shows that benders have a CHC profile intermediate between workers and queens (canonical plot for Fig. 15A).

The emergence of a queen-specific profile in future reproductives

In Colony 6, which lacked young females and pupae, a subset of middle-aged to old workers began bending 2 days after queen removal. Many were sacrificed and others were allowed to continue bending and to develop their ovaries for another week, so long as they were not repressed and demoted by their nest mates. Workers who initiated bending did not show a distinct profile from workers sacrificed alongside them or on the days prior (during queenright conditions) (Fig. 15B and Table 2). A week after queen removal, both workers and established benders showed a change in their CHC profile, with benders showing a more queen directed shift. The two failed benders from this colony (see above) grouped according to their behavior at the time of collection: a former bender who was observed working had a queenless worker profile whereas the active bender who was chased and attacked grouped with the cohort of established, accepted benders (Fig. 15B).

For Colony 8, established queens and, subsequently, young replacements queens (10-12 days old) were sacrificed. Unlike benders sacrificed from other colonies, these benders had begun laying eggs, and the majority of them were observed to fly off the nest near dusk, perhaps in search of mating opportunities. A discriminatory analysis based on all 22 compounds (PCA identified all compounds as contributors) was performed for queenright workers (>4 days old), queens and established benders from Colonies 6-8. The replacement queens from Colony 8 exhibit a CHC profile more queen-like than established, pre-queen benders from Colonies 6 and 7 (Figure 15C).

Queenright females treated with methoprene exhibit queenless CHC profile

Methoprene-treated workers from Colonies A and B were compared with their cyc- and non-treated counterparts. The PCA eliminated one variable, Z C30, and its exclusion from the discriminatory analysis had little effect on the results (with and without PCA: Global Wilks' λ=0.185; $F_{13,51}$=17.3; P<0.001; 100% predicted classification) (Fig. 16A). As expected, the difference between treatment groups was even greater when Colony A and B were analyzed separately since colony-specific contributions to CHC profiles are avoided (Colony A: Global Wilks' λ=0.093; $F_{10,26}$=25.4; P<0.001; Colony B: Global Wilks' λ=0.060 $F_{13,14}$=16.8; P<0.001. Importantly, when females from each nest were regrouped according to ovary size, irrespective of treatment, the differences were less evident (Colony A: Global Wilks' λ=0.650; $F_{5,37}$=4.0; P<0.001; 81% classification; Colony B: Global Wilks' λ=0.182; F=25.4; P<0.001; 95% classification).

A discriminatory analysis (after PCA elimination of 3 compounds) including established benders and queenright workers from Colonies 6 and 7 revealed that methoprene-treated females possess an established bender-like CHC profile (Figure 16B) while non-treated females were nested within the cyc-treated workers.

Discussion

Juvenile hormone is elevated in queens of *S. surinama*

In the present study, I show that JH titers are consistently higher in queens than workers in *S. surinama* (Fig. 4-5), suggesting that JH has gonadotropic, behavioral and/or signaling functions in queens, much like in *Polistes* (Izzo et al., 2010; Roseler, 1985; Tibbetts et al., 2011a). Consistent with earlier impressions (West-Eberhard, 1977) and contrary to the social organization of primitively eusocial paper wasps (e.g., *Polistes*, *Mischocyttarus*) (Noda et al., 2001; O'Donnell, 1998; Pardi, 1948; West-Eberhard, 1969), queens of *S. surinama* do not appear to rule according to a linear dominance hierarchy. If a hierarchy were present, winners would be expected to benefit by laying more eggs. However, high fecundity and low fecundity queen showed no difference in JH titers (Fig.4E), indicating that JH does not correlate with reproductive dominance in normal nests. Moreover, the JH titer of a defeated queen (who was attacked) was within range of normal queens. Taken together, these data suggest that JH is important for queen physiology but does not relate to dominance within a cohort of queens.

JH is a follower, not a leader, of aggressive interactions in prospective queens of *S. surinama*.

On queenless nests, young females (when present) became the next cohort of queens. When a colony lacked young females, old workers with filamentous ovaries were able to make the switch, demonstrating that caste-totipotency is not lost with age. Prospective reproductives signaled their physiological transition toward the queen phenotype by bending their abdomen, an act of ritualized aggression which, if evolutionarily stable, must be backed by outright aggression (West-Eberhard, 1977, 1983). Indeed, the act of abdomen bending resembles a stinging posture (Fig. 1D) and, like other forms of ritualized aggression (e.g., male-male assessments), must be derived from a non-ritualized proof of dominance, that is, fighting ability.

In general, benders of *S. surinama* had higher JH titers than their worker or idle counterparts who did not bend (Figs. 4 and 5). Yet it was evident from some nests that the JH titer elevated only *after* the onset of displaying. For example, callow females who were observed to bend within an hour of emergence had very low JH titer values (Fig. 4F). Benders in queenright conditions also had very little JH, and a rise was only seen in benders who were present when the last queen was removed (Fig. 4G). Furthermore, the variance of JH titers among old workers who transitioned into benders, some of whom had non-detectable amounts of JH, also suggests that an upsurge of JH lagged behind bending (Fig. 4D). Finally, I demonstrated that JH acts downstream of the switch to bending in two queenless situations. In the first case, old workers who were treated with JH showed no difference in their behavior from controls. Surprisingly, all females failed to initiate bending despite their putative totipotency, foregoing their own opportunity for direct reproduction. They may have autoregulated in response to the presence of pupae which would soon become adults with partially developed ovaries, a phenomenon observed in bumble bees (Bloch et al., 2002). (In Colony 6, where many old workers initiated bending in response to queen removal, there were very few pupae present.) JH treatment also had no effect on young females who eclosed in a period where caste fate was ambiguous (i.e., JH did not provide a boost in competitiveness in unstable circumstances) (Fig. 10).

In *Polistes*, where physical aggression is the proof of dominance, JH is typically higher (or their corpora allata are larger) in winners among competing reproductives (Roseler, 1985; Roseler et al.,1980; Tibbetts et al., 2011a). In the communal eumenid wasp *Zethus miniatus*, where females kill each other's offspring in order to procure rather than build empty cells, JH titers were decreased only when competition for cells was reduced (Chapter 2). In the swarm founding epiponine *P. micans*, JH spikes in young females who activate their ovaries and endure through a barrage of attacks from nest mates (Chapter 3). Thus, *S. surinama* stands

alone among studied social wasps by not providing any evidence that JH is important for aggression in the context of reproductive competition.

Hemolymph ecdysteroids are not important in *S. surinama*

Other candidate drivers of aggression are the ecdysteroids. In *Polistes*, the ovaries release ecdysteroids into the hemolymph (Roseler et al., 1985), and injection of 20E increases a foundress' chance of becoming dominant (similar to JH) (Strambi, 1990). This mechanism is not conserved in *S. surinama* since hemolymphal ecdysteroids were low and indistinguishable between queens, benders and workers (Fig. 9C). Therefore, an unknown factor incites aggressive displays in *S. surinama.*

The role of JH in queen-signaling

Queens and workers of *S. surinama* show obvious differences in their CHC profiles (Fig.12-13), and predictably, queen replacements (i.e., established benders) had a CHC profile similar to queens (Fig. 15). Most of these females have elevated JH titers, and so it is likely that JH conveys fertility information by way of chemical signaling rather than dominance displays. In support of this, JH application to queenright workers led to the acquisition of a CHC profile similar to that of incipient queens (Fig. 16B). The induced signal of fertility probably led their attack by fellow nest mates, although it is possible that other pheromones were co-effected (e.g., mandibular glands which likely emit queen pheromones; West-Eberhard, 1977). Initially, methoprene-treated wasps were mandibulated, solicited for proteinaceous saliva via trophallaxis and sometimes mounted by the aggressor. These intermittent, non-vicious attacks are occasionally observed on nests with young females, indicating that the experimental wasps were not attacked based on a foreign odor (also, treated females were not observed to be attacked on the day of initial treatment). As has been suggested in other social Hymenoptera (e.g., paper wasps (Reeve, 1991; West-Eberhard, 1996), honeybees (Visscher and Dukas,

1995)), the objective of such interactions is likely to curb ovarian maturation and/or stimulate working in prospective reproductives (Reeve, 1991). In methoprene-treated workers of *S. surinama*, the CHC profile continued to change toward a reproductive-like profile despite the attacks, likely resulting in the uninhibited aggression which eventually led to their permanent eviction from the colony.

Does JH directly affect the CHC profile of *S. surinama*? Or might JH stimulate ovarian development which in turn directs the production of fertility indicating factors on the epicuticle? The fact that some solvent-treated females, who were never attacked, had ovaries larger than some methoprene-treated females, who were always attacked, suggests that the ovaries are not as important as JH in CHC signaling. Also, grouping based on treatment (methoprene vs. solvent) resulted in a better separation than oocyte length in both colonies studied. Moreover, mandibulation attacks were observed a day after treatment, scarcely affording sufficient time for significant ovarian growth in the attackees. These results suggest a direct role of JH in modulating the CHC profile.

In *Polistes*, JH has been proposed as a mechanism for coordinating an individual's reproductive state and CHC profile (Izzo et al., 2010; Sledge et al., 2001). Interestingly, when fertility and dominance were disentangled in a study of *Polistes* foundresses, only fertility (i.e., ovarian state) showed a strong relationship with the CHC profile (Izzo et al., 2010). JH likely fails to induce fertility signaling in under-nourished or worker females (instead, it triggers precocious working behaviors) (Shorter and Tibbetts, 2009; Tibbetts and Izzo, 2009), indicating that the condition of the female is an important variable for JH function. The same may be true in *S. surinama* since old workers on a queenless nest were not observed to be attacked. *Polybia* appears to have lost the tight association between JH and chemical fertility-signaling. In *P. micans*, where queens and workers have distinct CHC profiles, JH is low in normal, oligarchic queens, and so JH is not needed to maintain the production of a queen signal (Chapter 3). In *P.*

occidentalis, methoprene-treatment of young queenright females induced changes in behavior but there was no report of subsequent attacks (O'Donnell and Jeanne, 1993).

JH as a gonadotropin

There are multiple lines of evidence suggesting that JH functions as a gonadotropin in *S. surinama*: 1) The upsurge of JH following the onset of bending in *S. surinama* is concomitant with ovarian maturation (Fig. 4), 2) JH titers correlated with oocyte length in most colonies (Fig. 7), 3) in one of two nests studied, JH rose slightly in queenright females as their oocytes lengthened in the few days following eclosion (Fig. 6B-C), and 4) JHM-application led to an increase in oocyte length (Fig. 11). The latter treatment results should be interpreted with some caution because of various interfering social factors. For example, methoprene-treated females became so harassed that they tended not to work, whereas control females participated in nest construction, an energy demanding task. Also, the difference in oocyte length between the two colonies tested was obvious and probably related to the number of queens. The monarchic colony featured females with larger oocytes, and these females showed a greater response to JH treatment than the colony with 4 queens. Thus, it is possible that queen number (e.g., pheromones) can limit the gonadotropic effects of exogenous treatment of JHM. The next step would be to repeat the JH treatment studies in a laboratory setting, if possible, where social factors can be removed and the duration of the experiment extended (as is done in *Polistes* (Tibbetts and Banan, 2010).

JH is gonadotropic in *Polistes* (Roseler, 1985). In *P. micans*, however, JH appears to only initiate ovarian activity and is not required for the maintenance of oocyte maturation, although it may augment ovarian growth in lone queens (Chapter 3). In *P. occidentalis*, methoprene was shown to induce precocious task performance in young queenright females, suggesting that JH does not have a gonadotropic function in young queenright females (O'Donnell and Jeanne,

1993). Thus, in terms of JH function in potential and actual reproductives (gonadotropic and CHC signaling), *S. surinama* appears to be more *Polistes*-like than *Polybia*-like.

JH in workers

Workers of *S. surinama* exhibit a clear temporal polyethism. Young queenright females were idle for the first days following eclosion. By day 2-4, they were observed to build, and I never observed a female forage within 10 days of eclosion. In most colonies studied, young, middle aged and old workers had very low JH titers. Yet in colonies where only pieces of the nest were removed (which did not lead to an obvious colony-wide re-allocation of worker tasks), some old foragers had elevated titers (Fig. 8). The scatter of JH titer values may reflect a rise and fall of JH titers associated with task transition, but because the individual histories of the females were not tracked, it is impossible to conclude whether or not JH is involved in age-related changes in behavior in *S. surinama*.

There is some evidence for a role of JH in regulating temporal polyethism in other wasps. Most notably, methoprene treatment of young females of *P. occidentalis* led to precocious transitions in age-related tasks (O'Donnell and Jeanne, 1993). In one colony of *P. micans,* relatively old foragers had higher JH titers than young builders (Chapter 3). Studies on *Polistes* suggest both reproductive and worker-modulatory functions operate within the same species (Giray et al., 2005; Shorter and Tibbetts, 2009; Tibbetts and Izzo, 2009; Tibbetts et al., 2011b). In well-nourished females, endogenous JH regulates reproduction and aggression (Roseler, 1985; Tibbetts et al., 2011b) while methoprene application to young, often underfed queenright females triggers precocious age-related behaviors (e.g., foraging in *P. dominulus* (Shorter and Tibbetts, 2009) and guarding in *P. canadensis* (Giray et al., 2005). Yet when JH titers are measured in foragers of *P. dominulus*, they are low (Tibbetts and Huang, 2010), and the higher titers of guards in *P. canadensis* (Giray et al., 2005) could be attributed to the known link between JH and aggression (and not working per se) (Roseler, 1985). Thus, although JH has

not been conclusively demonstrated to drive worker behavior in any one species, the evidence is suggestive across a number of eusocial wasp species.

Age- and social context-related changes in cuticular hydrocarbon profiles

In social insects, CHCs convey important information regarding age, reproduction, sex, nest membership and more (Blomquist and Bagnères, 2010). Callow females of *S. surinama* have a very distinct CHC profile that becomes more worker-like or queen-like, depending on the social context of the nest (Fig. 14). This is best illustrated with the dynamics of Z C25, an alkene which is always higher in queens than workers. Eclosing females have up to 40% representation of this single CHC. In a queenright nest, it drops precipitously to ~5% in workers whereas under queenless conditions it stays above 15% (Fig. 13-14). On occasion, freshly eclosed females received the queen dance from passing workers, and it is tempting to attribute this elicitation to the high amount of Z C25.

As was seen in the CHC profiles of callow *P. micans* (Chapter 3), newly eclosed *S. surinama* also possessed high amounts of relatively small hydrocarbons that drastically reduced over the first few days of adult life (Fig. 14). Workers from both species experienced a proportional rise in the longest hydrocarbons detected, while prospective reproductives with well-developed ovaries saw a rise in hydrocarbons associated with queens (the compounds of which were not shared between the species). Linear alkanes were detected in both species, but *S. surinama* was characterized by many more alkenes than *P. micans*.

Concluding remarks

In the first hormone study on highly (i.e., obligatory) eusocial wasps, methoprene was applied to young wasps of *Polybia occidentalis* who transitioned to worker tasks at an earlier age than control females (O'Donnell and Jeanne, 1993). As the authors noted, the lack of endogenous hormone measurements called for some reservation in their conclusion that JH regulates

temporal polyethism but not reproduction. Indeed, Zera (2007) articulates a convincing argument with numerous examples from the literature that studies which rely entirely on hormone treatment studies (divorced from endogenous measurements) should be viewed with suspicion. Here, 20 years later, I provide the first measurements of endogenous hormones in obligately eusocial wasps, and reveal that the endocrinology of eusocial wasps in general is much more diverse than anyone had expected. It is unclear what is more striking: the differences between *Polistes* and the two swarm founding species, or the differences between the two swarm founding species, *S. surinama* and *P. micans*. Table 3 summarizes the main findings in comparison to *Polistes*.

Hormone & effect	*Polistes* sp.	*S. surinama*	*P. micans*
JH high during social competition	yes	no	yes
JH higher in queens	yes	yes	no
JH higher in lone (vs. many) queens	n/a	no	yes
JH sustains ovarian development	yes	yes	no
JH sustains CHC profile	yes	yes	no
JH & temporal polyethism	probably	suggestive	suggestive
Ecdysteroids active in hemolymph	yes	no	not likely
Ecdysteroids in ovaries	yes	yes	yes

Table 3. Summary of differences in the endocrinology of three genera of wasps.

Alas, there is no grand unifying pattern of hormonal regulation in eusocial wasps. Far from being dismaying, the diversity and apparent susceptibility to modification of social wasp endocrinology provides an outstanding opportunity for evolutionary research. There are over 200 species of swarm founding wasps, and morphologically discrete castes have emerged many times over, with some species featuring queens which are actually smaller than workers. Epiponini is also

not uniform in social behaviors. For example, a bending ritual has been described in non-reproductive workers, the function of which is as unknown as the underlying physiology (Chavarria and Noll, 2012). The endocrinology of swarming is also of interest, for example, to compare swarms which happen naturally (fission and emigration swarms) versus emergency swarms which occur in response to a threat (absconding swarms) (Jeanne, 1991). West-Eberhard, renowned waspologist, said it best: “wasps are a microcosm for the study of development and evolution” (1996). Further work on the endocrinology of wasps spanning the entire spectrum of sociality will be essential for navigation inside this microcosm. The Epiponini in particular are ripe for interrogation.

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Figures

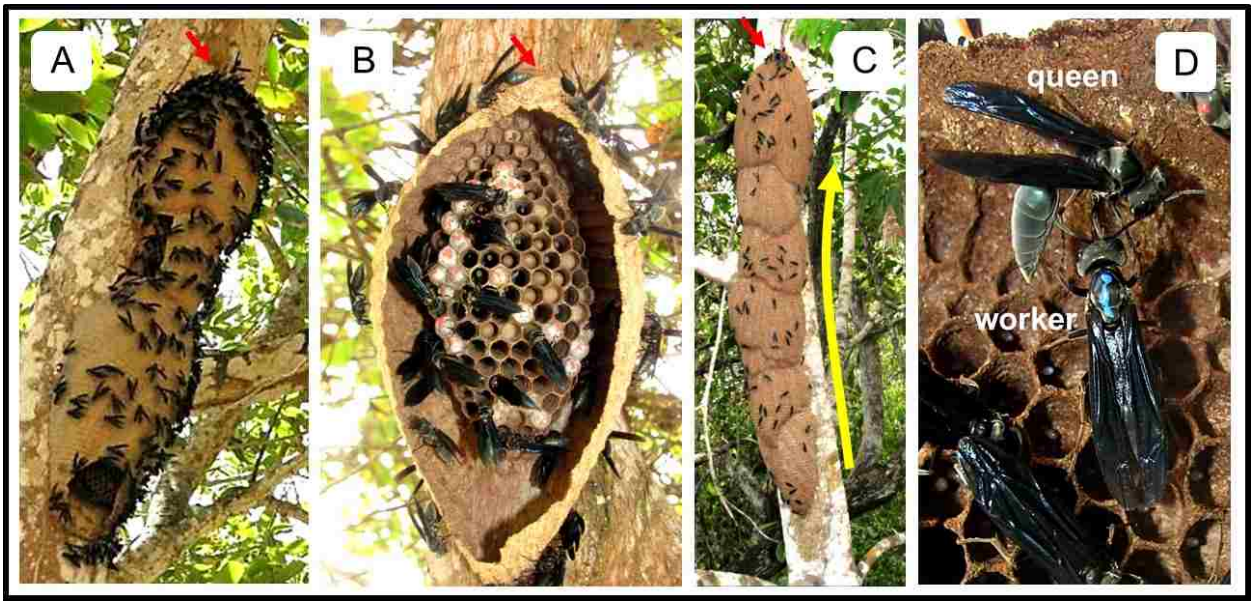

Figure 1: Nest architecture of *S. surinama*. (A) Aggressive defense response in a three compartment nest. The hole in the side was an observation window, and the opening on the bottom was incidental, leading to the emergence of colony defenders. (B) A single compartment nest with the envelope removed. Most nests were studied this way. (C) A massive nest discovered in Chapada Diamantina, Bahia, Brazil. Nest expansion proceeds upwards (yellow arrow). (D) Worker performs “queen dance” and the queen responds with a bending display. Red arrows indicate nest entrance/exit in A-C.

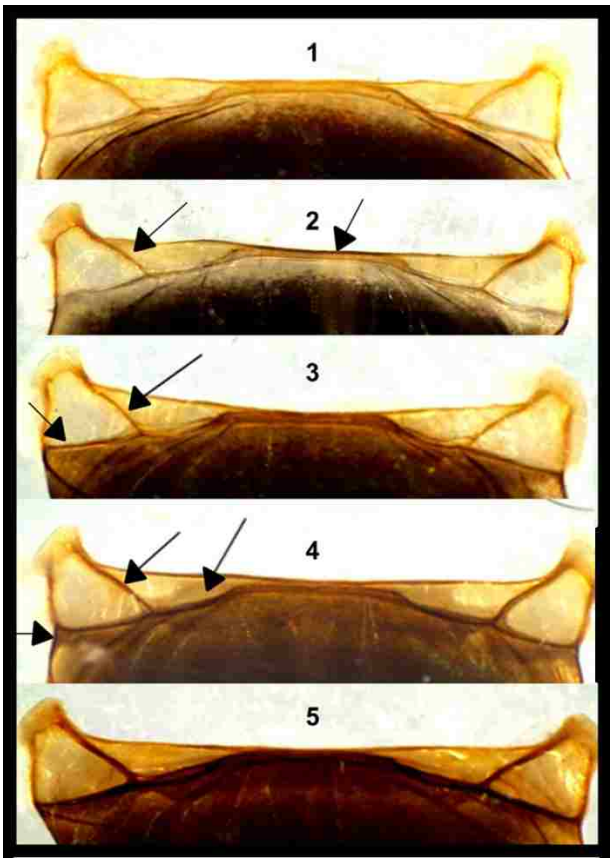

Figure 2: Relative age of individuals based on the analysis of apodeme darkening on the 5th abdominal sternite (1=new adult; 5=old adult). Arrows indicate areas of darkening.

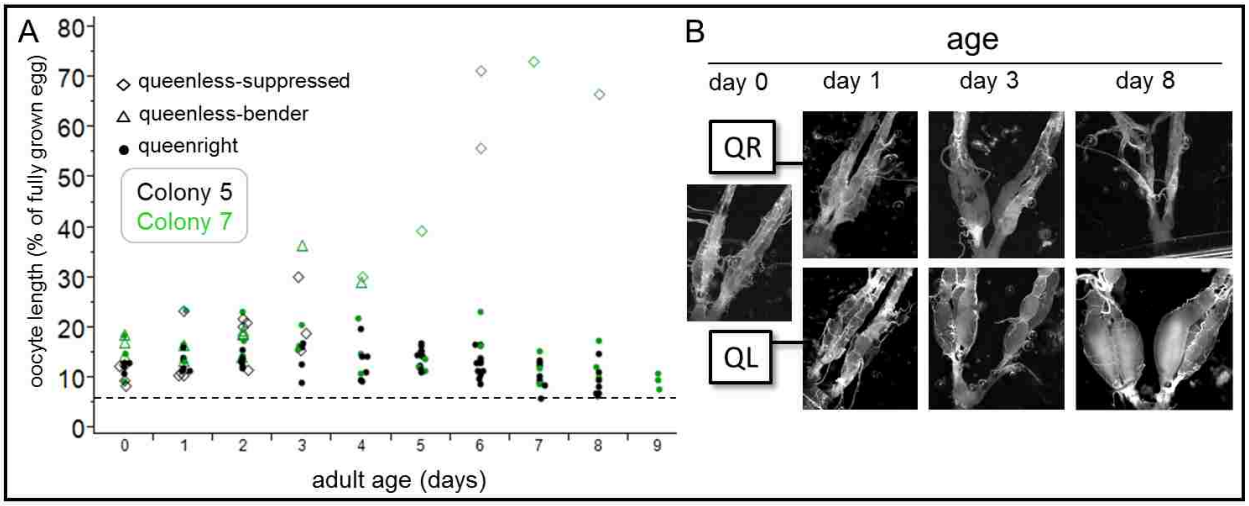

Figure 3: Ovarian maturation in young queenright and queenless females from two nests. (A) Newly eclosed females emerged with partially developed ovaries and experience limited oocyte growth in queenright conditions and pronounced ovarian development in queenless conditions. Dotted line indicates ovaries without visible oocytes. (B) Examples of ovaries from (A) which show the subtle growth and regression of oocytes in queenright (QR) conditions vs. the prominent growth observed in queenless females (QL).

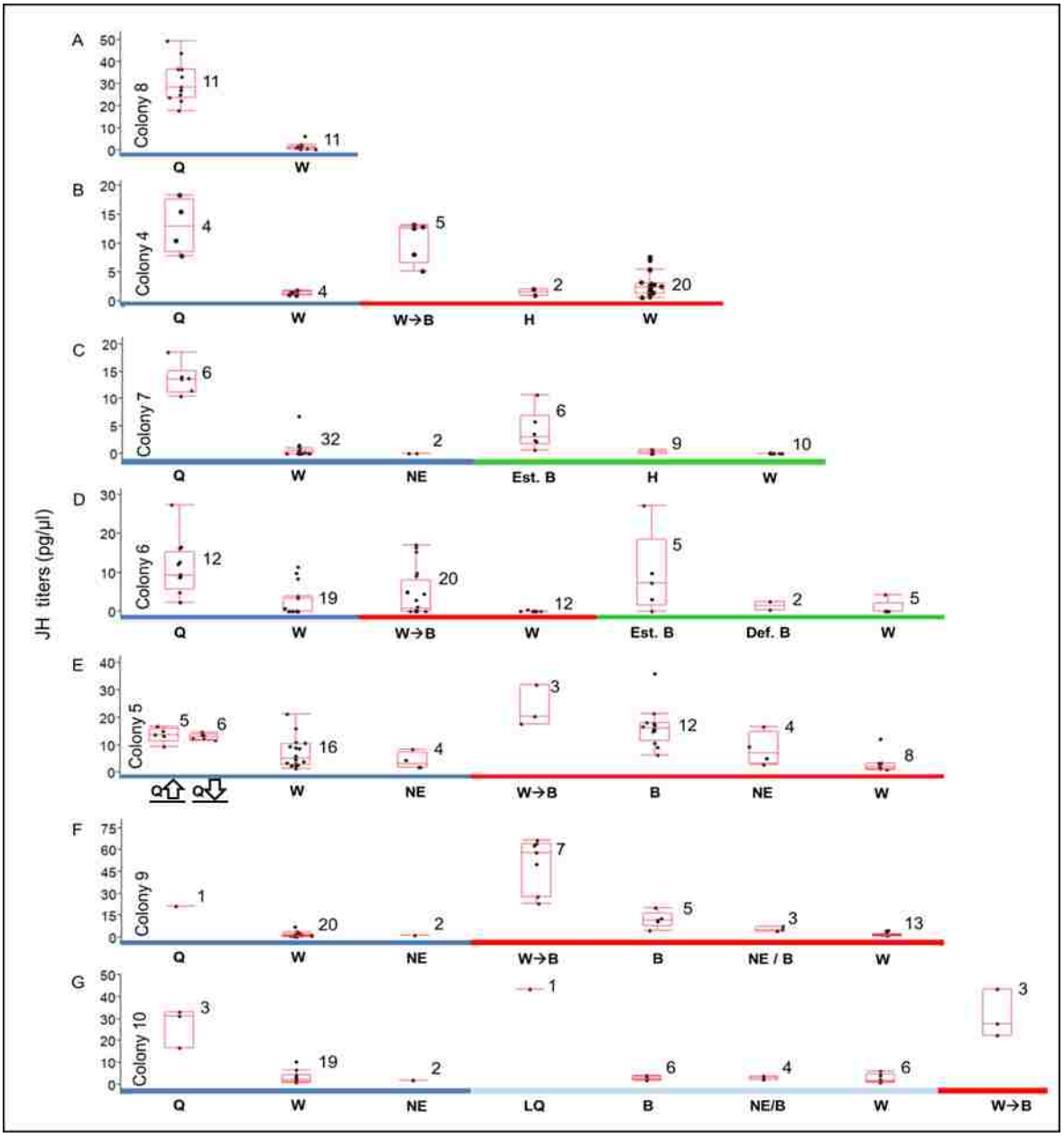

Figure 4. JH titers across all colonies. **Blue axis** indicates queenright (QR) conditions; **red axis** indicates queenless (QL) conditions within 2 days of queen removal; **green axis** indicates QL conditions 7-8 days after queen removal; **light blue axis** indicates QR conditions with 3 of 4 queens were removed. The latter is the only time when non-queens displayed in front of a queen. Box plots show the middle 50% of ovary scores and the whiskers indicate the 1.5 interquartile range. Key: Q=Queen; Q⇧=Queens with high fecundity; Q⇩= Queens with low fecundity; W=Worker; NE=Newly Eclosed female; H=Huddler (i.e., suppressed and idle females in QL nests); W→B=Worker-turned-Bender (i.e., they transitioned from working to displaying when the queen was removed); B=Bender (eclosed with no queens on nest and never worked); Est. B=Established Benders (i.e., incipient queens); Def. B=Defeated Benders (i.e., former benders working or being attacked); LQ=Lone Queen; NE/B= Newly Eclosed Benders.

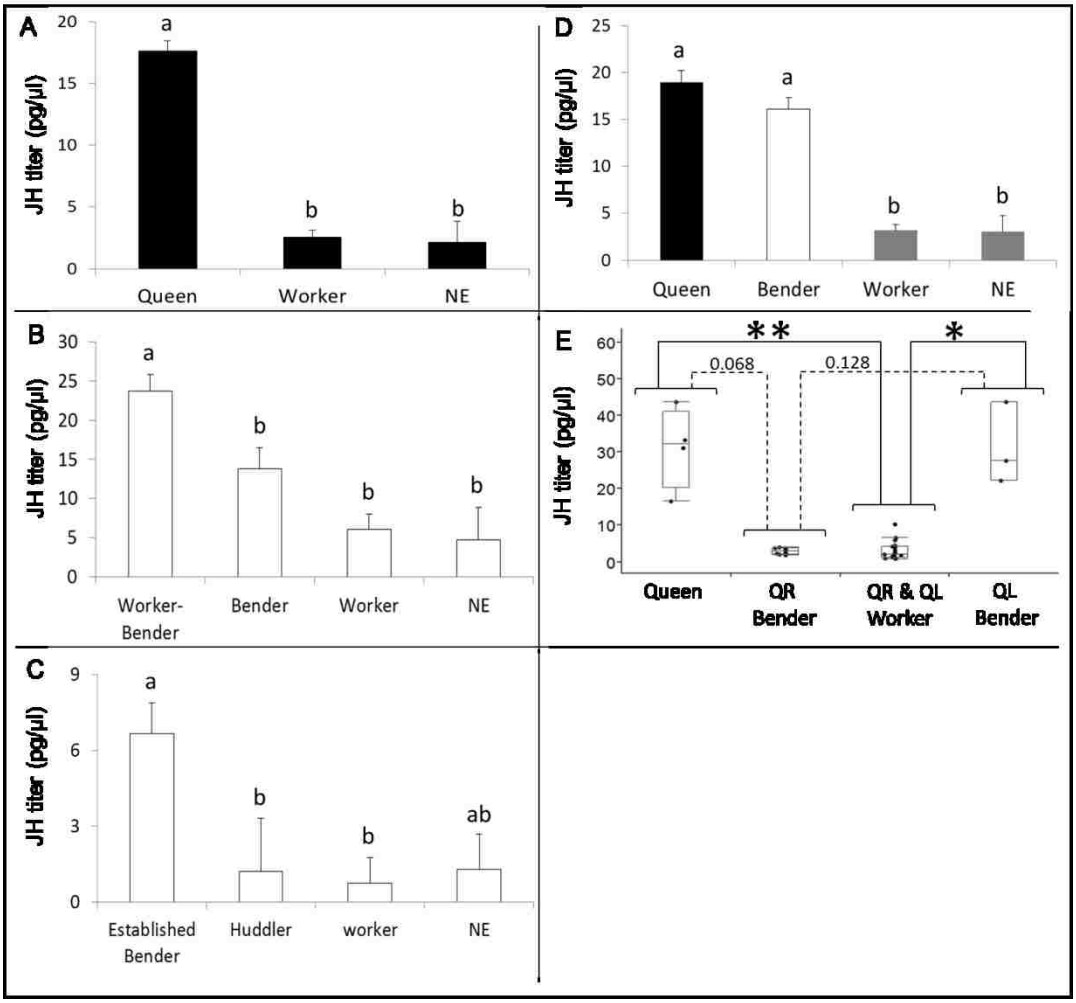

Figure 5. Pooled JH titers across all relevant colonies. Black bars indicate samples queenright conditions; white bars indicate samples in queenless conditions; and gray bars indicate a combination of samples from queenless and queenright conditions. (A) In queenright conditions, queens have higher JH titers than either workers or newly eclosed females. (B) In queenless nests 1-2 days after queen removal, workers who transitioned into benders in response to queen removal (Worker-Bender) had higher JH titers than younger benders who did not work prior to bending (Benders), workers and newly eclosed (NE) females. (C) In queenless conditions 7-8 days after queen removal, established benders (incipient queens) had higher JH titers than huddlers (suppresses potential reproductives) and workers but not newly emerged females (likely due to small sample size). (D) Queens and queenless benders (pooling both types of benders from 1-2 days after queen removal) had higher JH titers than pooled queenright + queenless workers and pooled queenright + queenless newly emerged females. (E) In Colony 10, queens and queenless benders had higher JH titers than pooled queenright + queenless workers (**P<0.01; *P<0.05). The sample sizes were not large enough to test for significant differences between queenright benders vs. queens or vs. queenless benders (All Pairs Steel-Dwass Method; a nonparametric version of Tukey Method). Statistics for A-E: Least squared means (±SE). A-C included two factors: Status and Colony while D included Colony Condition (queenright / queenless) as a third factor.

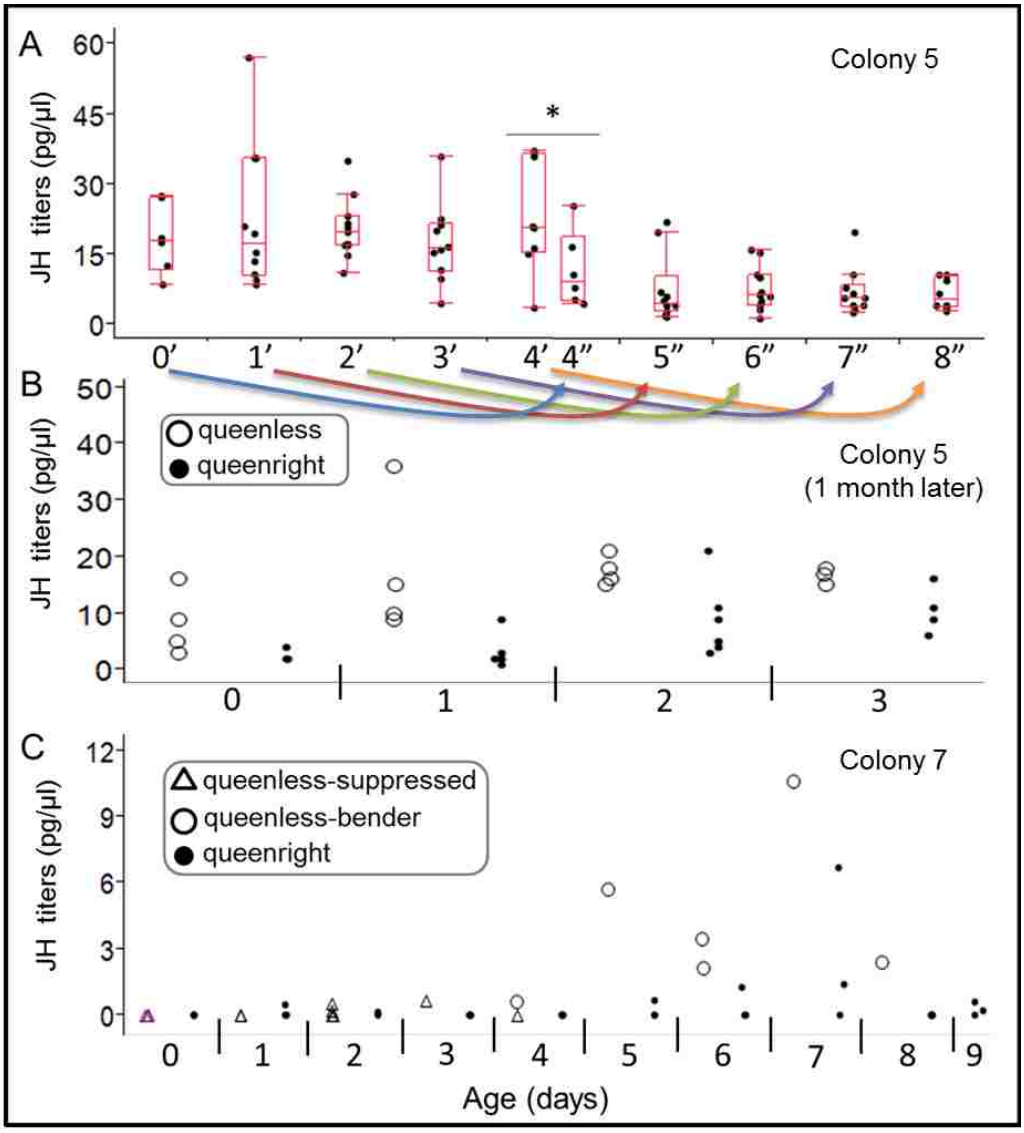

Figure 6: JH titers in young queenright and queenless females. (A) JH titers from females bled twice 4 days apart on Colony 5. For example, as indicated by the green arrow, females bled on day 2 after eclosion (day 2') were returned to the nest and bled 4 days later (day 6"). Females bled for the first time on day 4 (4') had higher JH titers than females bled a second time on day 4 (4"). Box plots show the middle 50% of ovary scores and the whiskers indicate the 1.5 interquartile range. (B) Queenright and queenless females taken from Colony 5 (at a later date than above). In general, queenless females (benders) appear to have higher JH titers, although both cohorts show a rise in the first few days following eclosion. When 1-3 day females are pooled according to colony state (QR vs. QL), queenless benders have higher JH titers than queenright workers. (C) Queenright and queenless females taken from Colony 7. Queenright females have very little if any detectable JH whereas queenless benders, fated to become the next queens, have scattered but relatively high titers. Younger queenless females, who emerged after the establishment of accepted benders, were suppressed and had low JH titers.

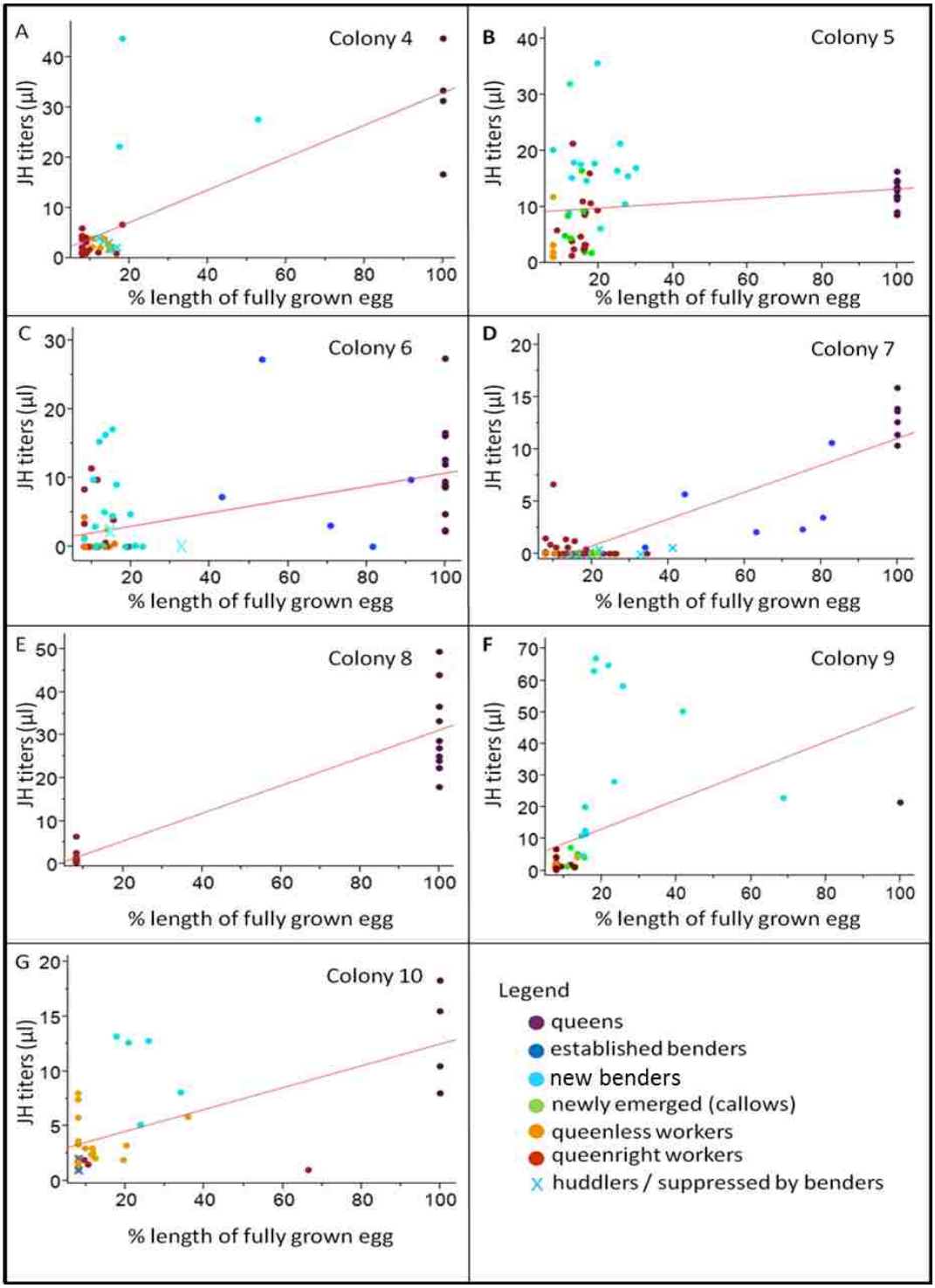

Figure 7: JH titers positively correlate with oocyte length (% length of fully grown egg) in most colonies of *S. surinama* (A, C-G), suggesting a gonadotropic function for JH. The presence of females who had recently initiated bending and were accepted (new benders in A-C, F-G) had higher JH titers than expected based on oocyte length, further supporting a role for JH in ovarian growth.

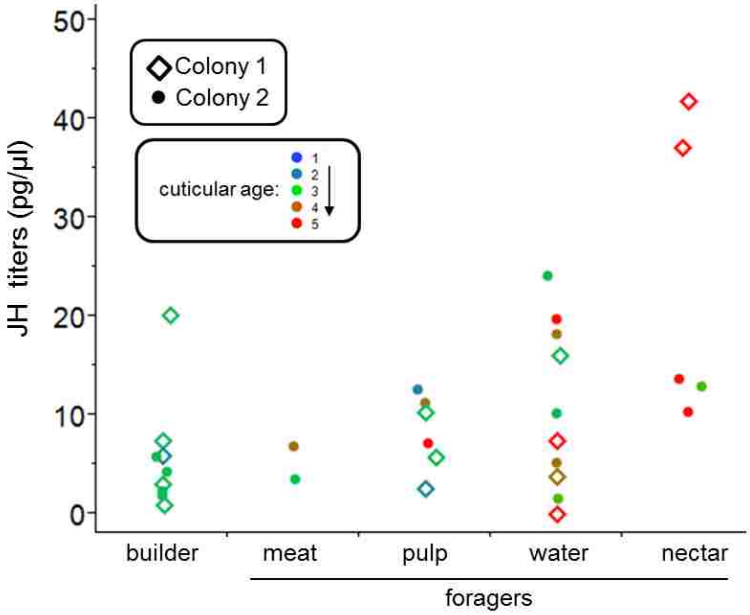

Figure 8: JH titers, age and task performed by workers from two colonies where the envelope was not removed. A cuticular age of 5 (Fig. 2) represents the oldest category of wasps.

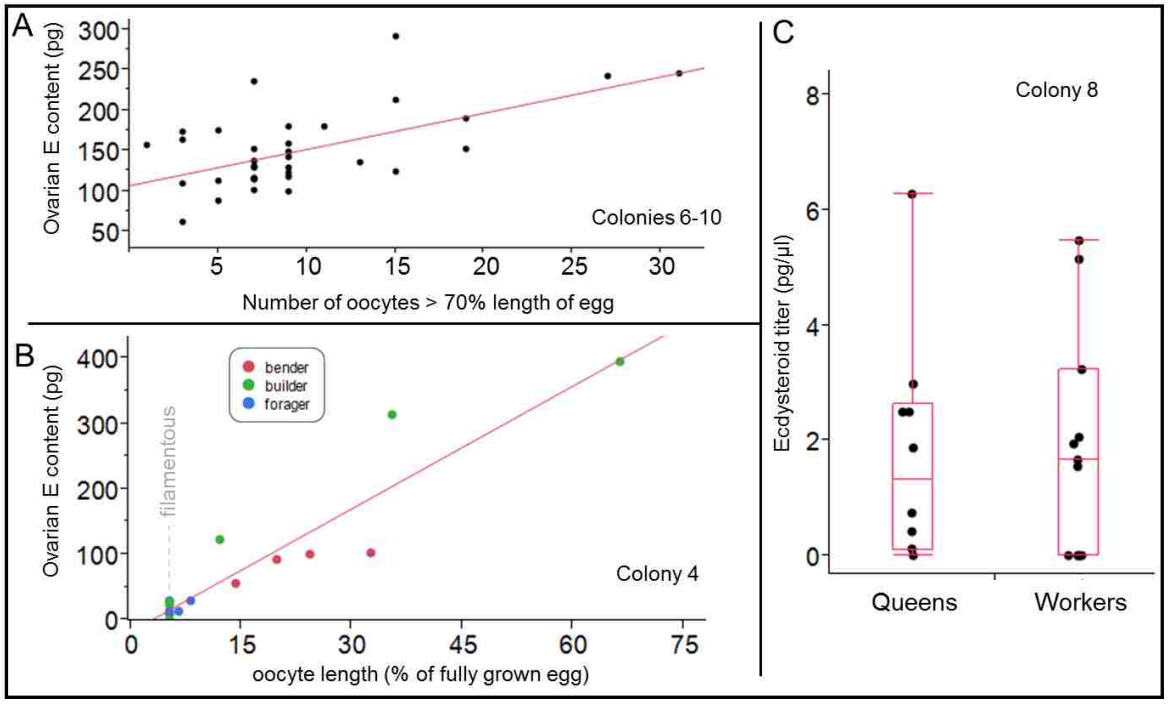

Figure 9: Ecdysteroids. Ovarian ecdysteroids correlate positively with (A) ovary size in queens from Colonies 6-10, and (B) oocyte length in workers and benders from Colony 4. (C) Hemolymph ecdysteroid titers did not differ between queens and workers. (Note: ecdysteroids from (A) and (B) were measured in separate RIAs, and the former is most likely an underestimate of ecdysteroid content; see Methods).

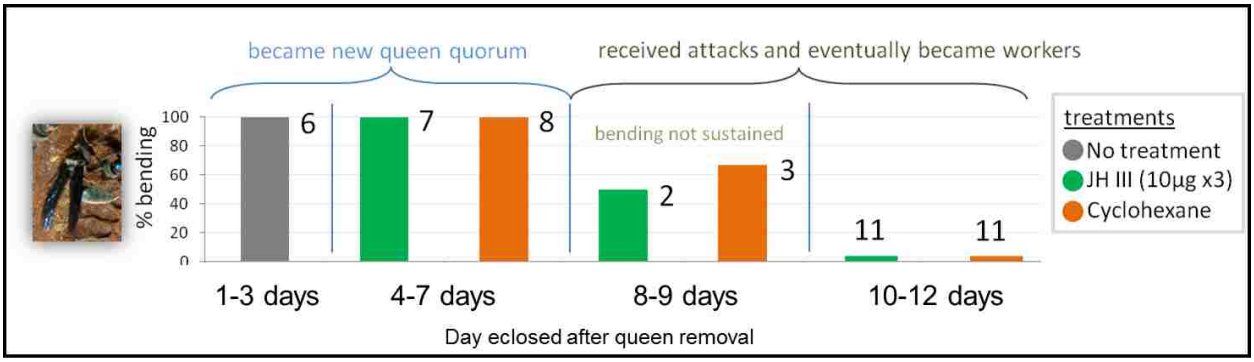

Figure 10: JH III treatment of young queenless females from Colony 8. The first 21 females to eclose following final queen removal became the next cohort of queens, irrespective of treatment. Females who emerged 8-9 days after queen removal engaged in bending behavior but were suppressed, eventually becoming workers. The following 22 females were attacked, were never observed to bend and all became workers. JH III treatment had no effect on a young female's ability to enter the cohort of queens or initiate bending.

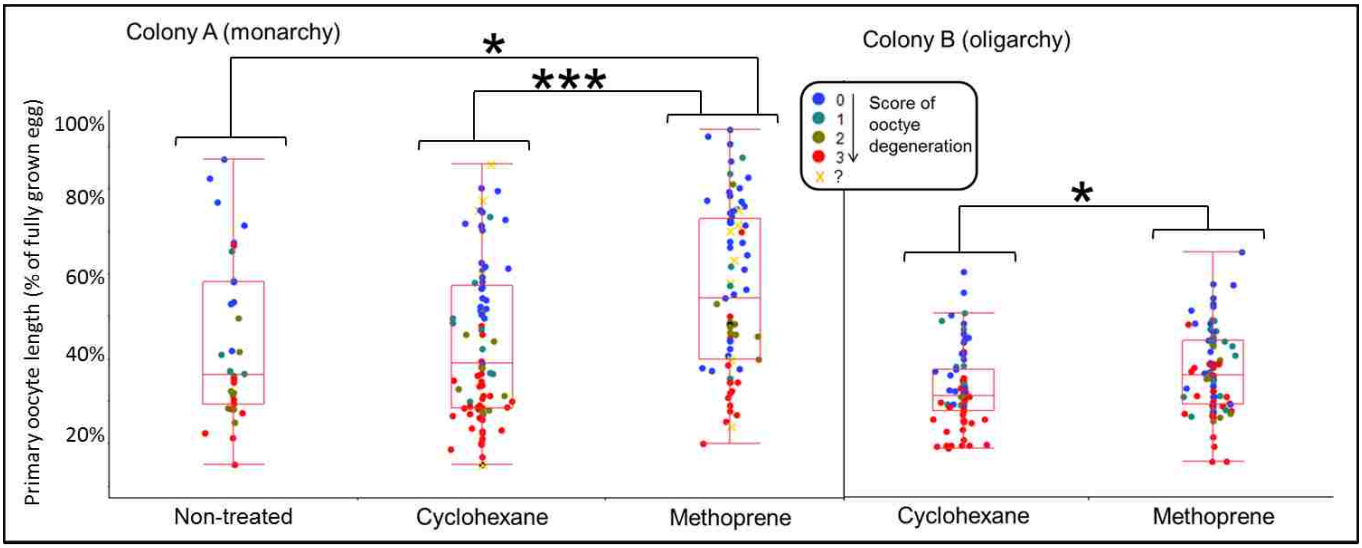

Figure 11: JH III treatment of young queenright females from Colony A (monarchic) and B (oligarchic). Each data point represents 1 of 6 measurable primary oocytes from treated and non-treated females. Color indicates state of oocyte degeneration. **P<0.001; *P<0.01

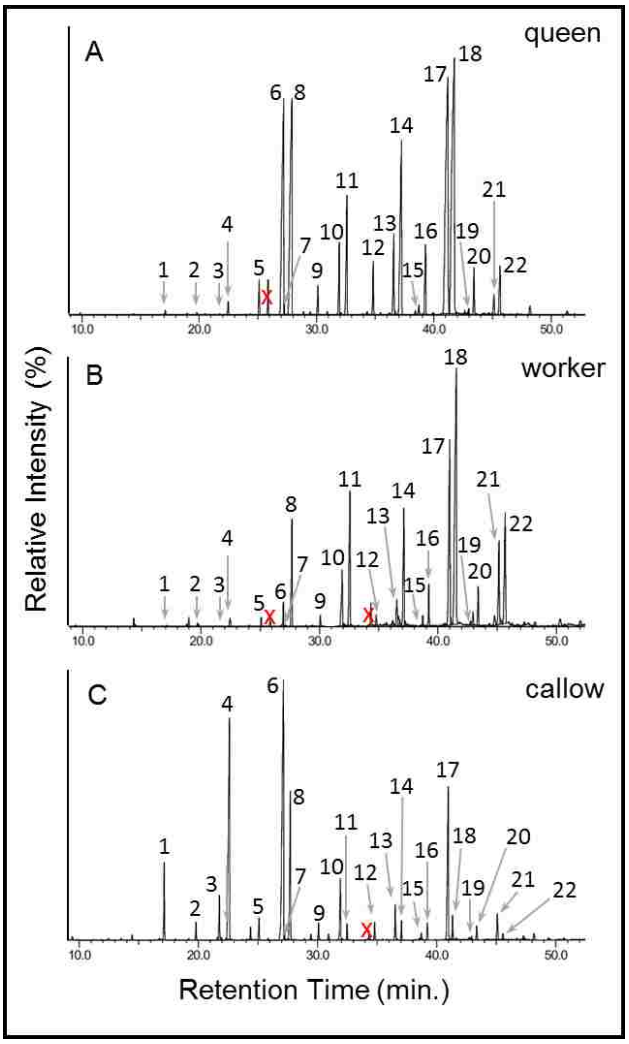

Figure 12. Chromatograms of whole-body extracts of queens, workers and callow females from *Synoeca surinama.* For compound numbering and identification, refer to Table 1. Red "x" indicates non-hydrocarbon or contamination.

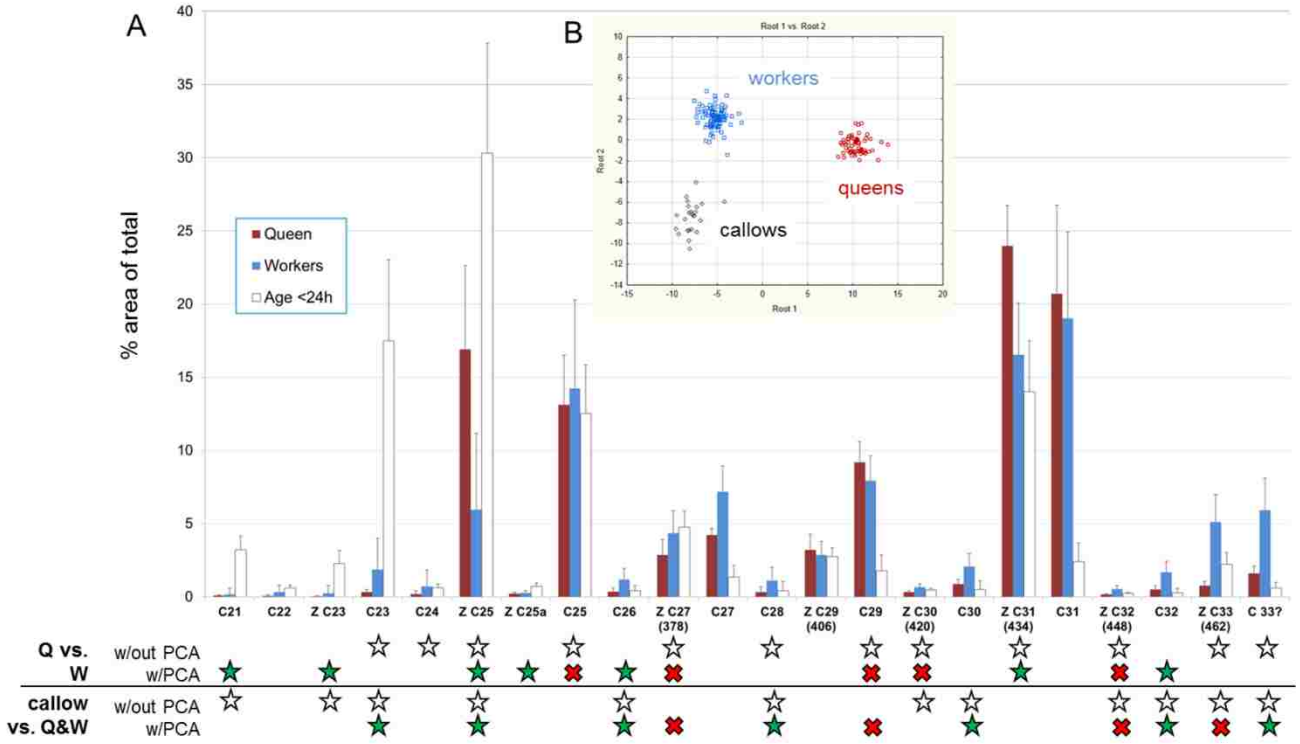

Figure 13. (A) Cuticular hydrocarbon profiles of queens, workers (>4 days since eclosion) and callow females from Colonies 4-8 (mean % ± SD). Discriminatory analyses were performed for queens vs. workers, and pooled queens+workers vs. callow females White stars and green stars indicate the main contributors of separation without and with PCA, respectively. Red X indicates the compound was removed based on the PCA analysis. (B) Canonical scatterplot based on a discriminatory analysis for all three groups.

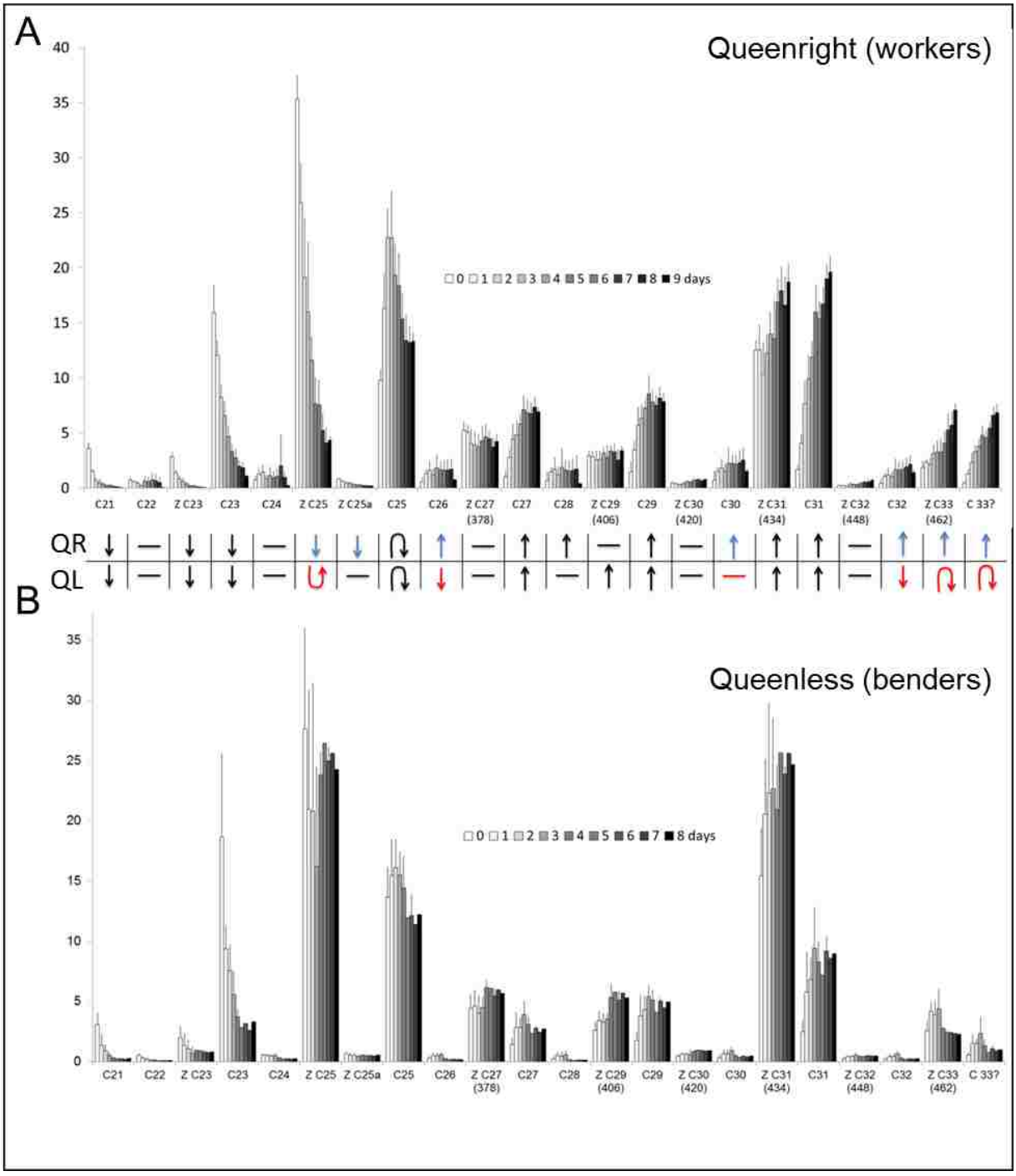

Figure 14. (A) Cuticular hydrocarbons of females who were sacrificed at 0-9 days since eclosion in queenright (top) and queenless (bottom) conditions from Colonies 5 & 7 (mean % ± SD). Middle rows of arrows indicate obvious changes in a compound's proportional representation over the first 8 or 9 days of adult life. Red arrows indicate a queen-like shift and blue arrows signify a worker-like shift.

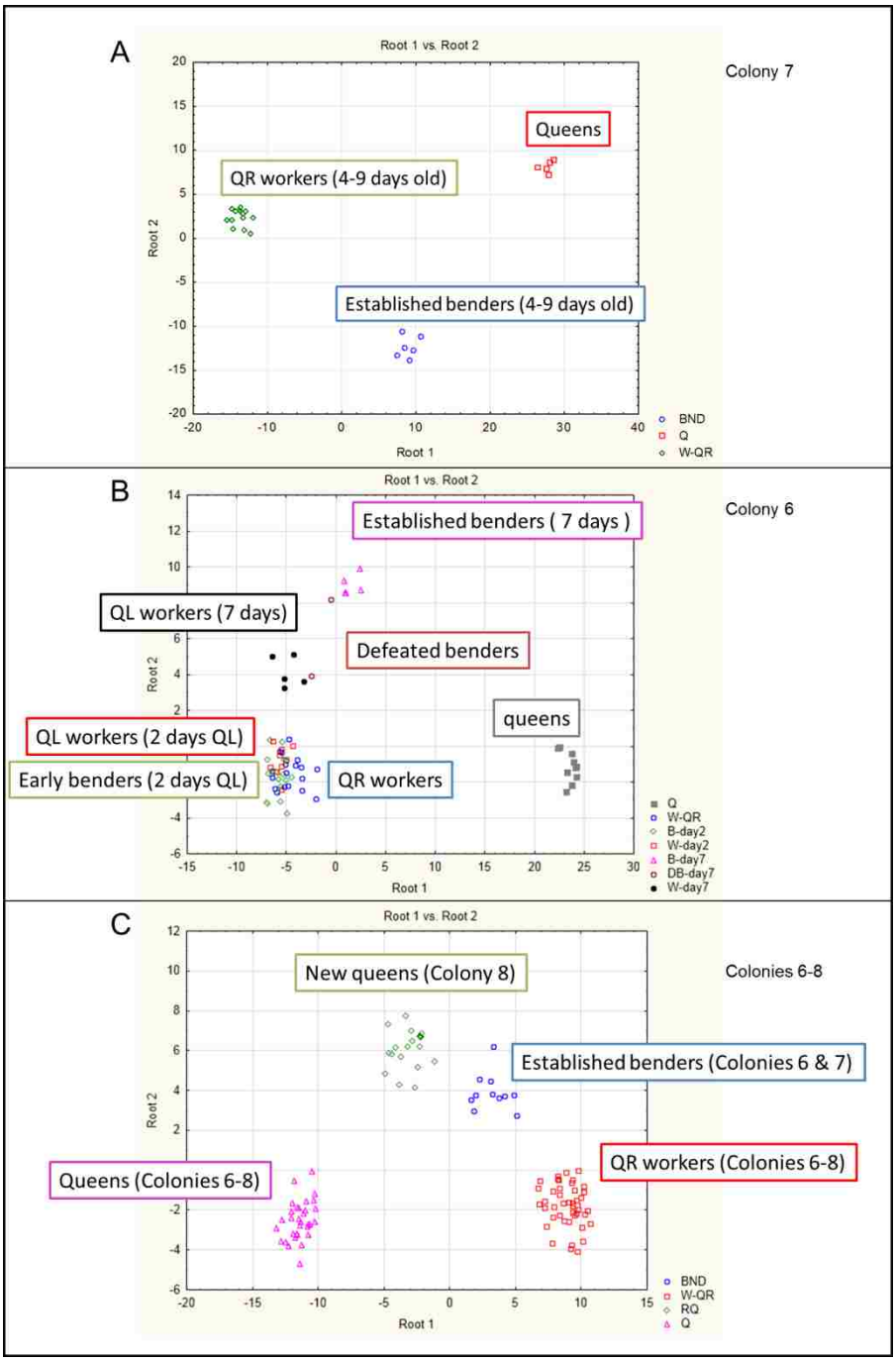

Figure 15. Established benders show a shift toward a queen-like CHC profile. The canonical scatterplots are based on a discriminant analysis including: (A) Queens, 4-9 day old adult queenright workers and comparably aged queenless females (established benders who are fated to become queens) from Colony 7. (B) Queens and queenright workers, queenless workers and benders 2 days after queen removal, and queenless workers, benders and two defeated benders 7 days after queen removal. All females were from Colony 6 and had eclosed over a month prior. (C) Pooled queens, queenright workers, established benders (i.e., benders for at least 4-9 days) and young queens removed from Colonies 6-8.

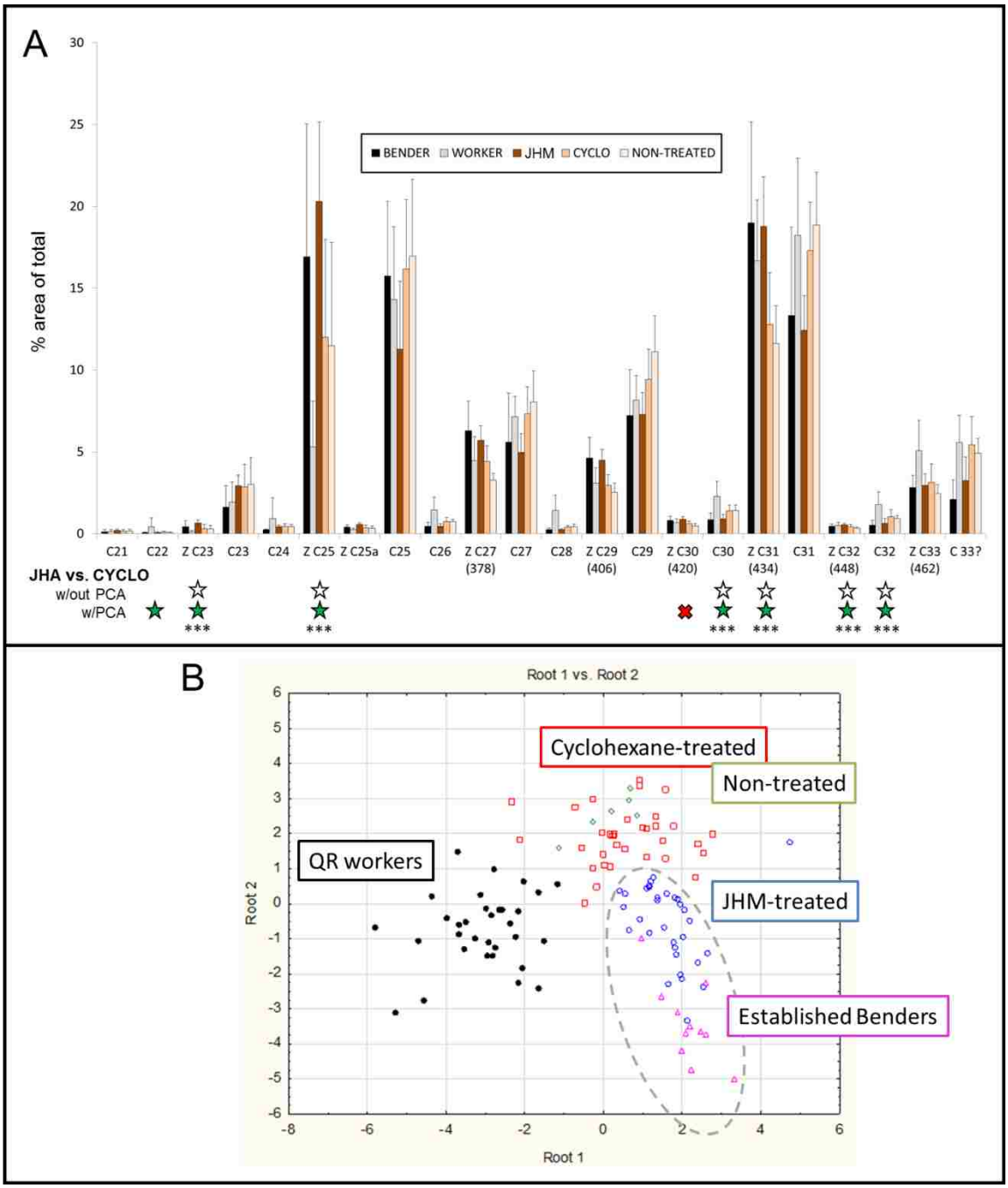

Figure 16. (A) Methoprene (JHM)- treated females produce distinct CHC differences from that of cyclohexane or non-treated females, and the differences are mirrored in established benders vs. queenright workers (from Colony 6 & 7). A discriminatory analysis of JHM-treated and cyclohexane-treated females: White stars and green stars indicate the main contributors of separation without and with PCA, respectively. Red X indicates the compound was removed based on the PCA analysis. (B) Canonical scatterplot based on a discriminant analysis including the above groups. Non-treated controls group within the cyclohexane-treated females, and methoprene-treated females overlap with the established benders.

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I dedicate this thesis to the memory of Robin Mariko Harris, one of the most beautiful and selfless people I have ever met. Your passing has never made me feel more sad… and human.

Releasing of *Manduca sexta* following Robin's memorial at Janelia Farm.