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Commentary

Muscle biochemistry and the ontogeny of flight capacity during behavioral development in the honey bee, *Apis mellifera*

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Summary

A fundamental issue in physiology and behavior is understanding the functional and genetic mechanisms that underlie major behavioral shifts in organisms as they adopt new environments or life history tactics. Such transitions are common in nature and include the age-related switch from nest/hive work to foraging in social insects such as honey bees (*Apis mellifera*). Because of their experimental tractability, recently sequenced genome and well understood biology, honey bees are an ideal model system for integrating molecular, genetic, physiological and sociobiological perspectives to advance understanding of behavioral and life history transitions. When honey bees (*Apis mellifera*) transition from hive work to foraging, their flight muscles undergo changes that allow these insects to attain the highest rates of flight muscle metabolism and power output ever recorded in the animal kingdom. Here, we review research to date showing that honey bee flight muscles undergo significant changes in biochemistry and gene expression and that these changes accompany a significant increase in the capacity to generate metabolic and aerodynamic power during flight. It is likely that changes in muscle gene expression, biochemistry, metabolism and functional capacity may be driven primarily by behavior as opposed to age, as is the case for changes in honey bee brains.

Key words: behavioral development, flight, aerodynamics, energetics, gene expression, reserve capacity, *Apis mellifera.*

Introduction

A central issue in physiology and ethology is understanding the metabolic and genetic mechanisms underlying major age-related shifts in behavior and life history. It is likely that the underlying mechanisms of such shifts are shared with or elaborated from mechanisms of neural and motor development, and behavioral plasticity in general, in an ecologically relevant manner across evolution. Furthermore, it is often the change in physiological capacity associated with these age-related shifts in behavior and life history that determines the adaptive value of the new behavior or life history tactic. Most research addressing this issue has focused on mechanisms and genes used by adult organisms during seasonal transitions in behavior (accompanying reproduction, migration, hibernation, estivation, etc.) or those used by sub-adult organisms during behavioral transitions accompanying environmental and/or resource shifts that occur during development. Much less studied are the underlying mechanisms of behavioral transitions that occur typically only once during the adult stage as an organism adopts a new environment or life history tactic. Such transitions are common in nature and often accompany increased physiological capacity for the behavior, such as the sex change that occurs with increased size or loss of a dominant individual in some groups of fishes (e.g. Demski, 1987; Cardwell and Liley, 1991). Other examples of adult behavioral transitions supported by shifts in physiological function include endocrine and environmentally induced insect polymorphisms (e.g. Applebaum and Heifetz, 1999; Pener and Yerushalmi, 1997; Rankin, 1991; Zera and Cisper, 2001; Zhao and Zera, 2002, 2004), the transition from saltwater to freshwater during salmon spawning migrations (Dittman and Quinn, 1996; Mommsen, 2004; Onuma et al., 2003), acquisition of song and associated changes in gene expression and neural structure in many species of passerine birds (Brenowitz and Beecher, 2005; Clayton, 2004; Mello et al., 2004) or the increased power output in flight muscle that accompanies dragonfly adult maturation (Fitzhugh and Marden, 1997; Marden et al., 1999, 2001).

Another classic example is the transition from nest work to foraging in social insects, which is the focus of this commentary. Understanding the physiological and genetic underpinnings of behavioral transitions requires the study of model organisms whose ethology, physiology and genome are simultaneously well characterized and experimentally tractable. At present, few model organisms satisfy all these

demanding criteria because most have yielded genetic and physiological findings without an understanding of their behavior in an ecological–evolutionary context or *vice versa*. However, the honey bee, *Apis mellifera*, is a model system whose experimental tractability is powerful and rapidly evolving, making this species among the best available for the study of social behavior and development. Indeed, honey bee evolution, behavior, physiology and genetics are each well represented in an abundant literature (>5000 references) on this species.

Honey bees are oviparous (egg laying), holometabolous (completely metamorphosing) insects that live in large colonies usually containing over 20 000 individuals. Embryos and larvae are individually housed in open cells on honeycombs and are cared for by adult bees. The environment of the brood is maintained by the bees and is remarkably constant. The temperature is maintained between 30 and 35°C, carbon dioxide levels are held between 1 and 4.3%, and relative humidity is regulated between 70 and 75% (Winston, 1987). Honey bees exhibit a form of behavioral development termed 'temporal polyethism', moving through a series of behaviorally defined life history stages in an age-related fashion. For the first 2–3 weeks of life, adult workers perform tasks inside the hive such as brood care ('nursing') and hive maintenance. Typically at about 3 weeks of age, workers transition to performing tasks outside the hive such as foraging. Foraging bees are typically the oldest workers in the hive. The physiology of honey bees changes as they age and move from non-flying tasks in the hive to foraging, which imparts a suite of different functional demands. For example, hypopharyngeal glands regress and produce enzymes for processing nectar instead of brood food, juvenile hormone levels increase, body mass decreases, body water content increases and, as we describe in more detail below, metabolic and flight capacity increases (Fluri et al., 1982; Harrison, 1986; Huang et al., 1994; Ohashi et al., 1996, 1999; Pontoh and Low, 2002; Robinson and Vargo, 1997; Winston, 1987).

Many behavioral transitions made as animals enter a new environment or life history stage in adulthood typically occur only once, but honey bee adult behavioral development is exceedingly plastic and responsive to the social environment; honey bees can move into a later stage precociously, delay the transition or return to a previous behavior (and the previous physiological state associated with that behavior) depending on the social context and colony needs. For example, in colonies deficient in nurses, young bees will continue to tend brood rather than switch to outside tasks (Robinson et al., 1989). Similarly, in colonies completely lacking young bees, older bees that would normally be foragers often revert to nursing behavior. In reverted nurses, juvenile hormone levels drop and their hypopharyngeal glands enlarge to resemble those of normally aged nurses (Huang and Robinson, 1996; Page et al., 1992; Robinson et al., 1992). The effect of colony demography on foraging behavior appears to be due to social inhibition, as the presence of older foraging bees inhibits foraging by younger bees. In colonies that lack a normal cohort of older foraging bees, younger bees begin to forage precociously – as early as 5 days of age (Huang and Robinson, 1992; Robinson et al., 1989). When normal-aged foragers are transplanted into a 'single-cohort' colony containing only young bees, precocious development of foraging does not occur (Huang and Robinson, 1992). In addition, adult behavioral development also varies with a colony's genetic background and is sensitive to factors such as weather, season, parasite infestation and colony nutritional status (Giray and Robinson, 1994; Giray et al., 1999; Huang and Robinson, 1995; Janmaat and Winston, 2000; Kolmes and Winston, 1988; Page et al., 1992; Schulz et al., 1998).

Honey bee behavioral genetics

Behavioral development in honey bees requires the same underlying processes such as learning, circadian rhythmicity, motor control, and sensory processing as it does in rats, mice or humans. Many of these processes have been studied at the molecular level in the brains of vertebrate models. These pathways are likely to be common to both vertebrates and invertebrates and, thus, many studies of honey bee behavioral development have focused on changes in gene expression in the brain. Among the genes in the brain known to be differentially regulated between foragers and nurses is the clock gene *period*, which is involved in regulating circadian rhythmicity, which honey bees acquire just prior to the onset of foraging behavior (Bloch et al., 2001; Toma et al., 2000). Foragers and nurses also differentially express genes involved in calcium and cholinergic signaling (Shapira et al., 2001; Takeuchi et al., 2002), as well as those coding for cAMP-dependent protein kinases, which control protein function and act in intracellular signaling during memory formation (Fiala et al., 1999).

Recently, cDNA microarrays developed from a honey bee expressed sequence tag (EST) database (Whitfield et al., 2002) have revealed different patterns of gene expression in forager *vs* nurse brains independent of age (Whitfield et al., 2003). Among the genes differentially regulated between behavioral groups are those with strong sequence matches to annotated *Drosophila* genes important in axiogenesis, cell adhesion and intracellular signaling. In the latter class is *foraging* (*for*), a previously identified cGMP-dependent protein kinase whose pharmacological activation causes precocious positive phototaxis and precocious foraging (Ben-Shahar et al., 2002, 2003). Similarly, a recent macroarray study demonstrates higher expression levels for genes involved in signal transduction, ion channels, neurotransmitter transport, transcription factors, plasma membrane proteins and most cell adhesion proteins in foragers as compared with newly emerged bees, suggesting plasticity and remodeling of neurocellular properties during aging and/or behavioral development in honey bees (Tsuchimoto et al., 2004). Using an earlier non-normalized version of the honey bee brain cDNA library later used to develop the bee EST database, Kucharski and Maleszka (2002) showed that foragers also increase the

expression of genes encoding royal jelly proteins, metabolic enzymes (α-glucosidase, aminopeptidases, glucose dehydrogenase) and a LIM domain protein that is a putative transcription regulator.

Gene expression in honey bee brains is also sensitive to environmental factors. Exposure to queen mandibular pheromone (which is known to promote brood care among hive bees) upregulates genes typically expressed by nurses working at brood care and downregulates genes whose expression levels are typically higher in foraging bees (Grozinger et al., 2003). These studies clearly show that gene expression in honey bee brains changes relative to behavioral task and help to identify some of the genetic and biochemical mechanisms underlying behavioral transitions in this species. An important remaining goal is to identify the suite of functional and genetic changes in other honey bee tissues, such as flight muscle, during the switch to foraging, a task that requires rates of metabolism and muscle power production that are among the highest ever recorded in the animal kingdom (see Roberts and Harrison, 1999).

Age-related changes in flight metabolism and muscle biochemistry in honey bees

Adult honey bees go from being unable to fly during the first day following eclosion to generating spectacular rates of metabolism and aerodynamic power (up to 0.8 W g^{-1} and 0.2 W g^{-1}, respectively; Roberts and Harrison, 1999) that enable later work outside the hive, traveling up to 8 km from the hive and carrying loads equivalent to their body mass during foraging and undertaking. The development of flight ability generally occurs in two distinct periods, the first being the 3–4 days following eclosion and the second typically at 14–21 days post-eclosion during the transition from hive work to foraging. 1-day-old bees that are physically agitated (a manipulation generally assumed to induce maximal metabolic capacity) can generate metabolic rates of only 0.1 W g^{-1} and are isothermic with the surrounding air. Hovering 2-day-old bees have metabolic rates approaching 0.3 W g^{-1} and are more endothermic; coincident with the increase in metabolic capacity of young bees are dramatic increases in thoracic pyruvate kinase and citrate synthase activities as well as thoracic glycogen levels (Fewell and Harrison, 2001; Harrison, 1986; Harrison and Fewell, 2002; Moritz, 1988; Neukirch, 1982). Such biochemical changes should, in theory, greatly increase flux capacity through the citric acid cycle and the rate of NADH and $FADH_2$ recycling. Flight metabolic rates, thoracic enzyme levels and thoracic glycogen levels remain relatively constant over the 1–3-week period when the bees work within the hive. Then, at the onset of foraging (14–21 days post-eclosion), there is an approximate 15% increase in agitated flight metabolic rate, coincident with an approximate doubling of thoracic glycogen levels (Fewell and Harrison, 2001; Harrison, 1986). Further enhancing oxidative capacity in honey bee flight muscle is a 10-fold increase in cytochrome concentrations from 1 to 20 days after eclosion (Herold and Borei, 1963). Similarly, cytochrome *c* oxidase activity increases twofold during flight muscle maturation in adult grasshoppers (Sogl et al., 2000). Variation in insect flight metabolism and/or performance has been linked to allozyme variation in several enzymes involved in cellular respiration, including phosphoglucose isomerase (Watt, 1992), glycerol-3-phosphate dehydrogenase (Barnes and Laurie-Ahlberg, 1986) and malate dehydrogenase (Coelho and Mitton, 1988; Harrison et al., 1996b). In addition, quantitative trait locus (QTL) analysis in *Drosophila* has linked the metabolic enzymes glycogen synthase, hexokinase, phosphoglucomutase and trehalase activity to variation in metabolic rate and flight performance (Montooth et al., 2003). Hence, age and/or behavior-dependent variation in these and other metabolic enzymes may underlie the development of flight capability in adult honey bees.

Structural and regulatory proteins of the flight muscle may also be changing as honey bees age and transition to flight-dependent behaviors. Honey bees possess asynchronous flight muscle (AFM). Unlike synchronous muscles (which, like typical striated muscles, have a 1:1 ratio of neural stimuli to contractions, with contraction initiated by intracellular calcium release and terminated by calcium uptake by the sarcoplasmic reticulum), AFMs show an approximately 1:10 ratio of neural stimuli to contractions. Neural stimulation in AFM releases intracellular calcium that removes thin filament inhibition, but the cross bridges themselves are activated by stretch and deactivated by sarcomere shortening. AFMs are stretched by thoracic deformation caused by contraction of antagonistic muscles, and this mechanical feedback keeps AFMs contracting over many cycles (Josephson et al., 2000; Pringle, 1957; Tregear, 1977). The large, power-producing AFMs are controlled by a set of small synchronous muscles that produce little or no power but are capable of rapid and finely graded responses to neural stimuli (Dickinson and Tu, 1997; Dickinson et al., 1998). Troponin-T (TnT) is the tropomyosin-binding protein of the calcium-regulated troponin complex of striated muscle, and honey bees express different TnT isoforms in their thoraces at one-day *vs* five-day post-eclosion (Domingo et al., 1998). This result suggests that honey bees are altering their calcium-dependent regulation of muscle contraction in an age-specific manner consistent with the acquisition of functional flight capability. In flight muscles of the dragonfly *Libellula pulchella*, the mixture of TnT isoforms also changes during adult maturation, with correlated changes in calcium sensitivity of muscle activation, twitch contraction kinetics and other indices of aerodynamic power output during free flight (Fitzhugh and Marden, 1997; Marden et al., 1999, 2001). Studies of *Drosophila* mutants have elucidated the roles of numerous other genes involved in the structure and regulation of AFMs whose expression may vary in an age- or behavior-dependent manner in honey bees. These genes include those coding for myosin regulatory light chain (Moore et al., 2000; Tohtong et al., 1995), flightin (Ayer and Vigoreaux, 2003; Henkin et al., 2004; Reedy et al., 2000), paramyosin/miniparamyosin (Maroto et al., 1996), calcineurin (Gajewski

et al., 2003), kettin (Kulke et al., 2001) and tropomodulin (Mardahl-Dumesnil and Fowler, 2001).

Supporting the hypothesis that honey bee flight muscles undergo significant age and/or behavior-dependent biochemical changes is our recent observation that foragers express greater amounts of heat shock proteins (Hsps) in their thoraces relative to nurse bees (Fig. 1). Hsps are part of a larger suite of molecular chaperones that participate in the maturation, maintenance and degradation of diverse proteins in both unstressed and stressed cells and are nearly universal in organisms (Feder and Hofmann, 1999; Gething, 1997; Morimoto et al., 1994). We measured Hsp70 and other members of the 70-kDa family of molecular chaperones in honey bee heads and thoraces as a function of age/behavior (nurse bees *vs* older foragers returning from a trip). Foragers expressed more Hsp70 in their thoraces than nurse bees, although there was no significant difference in head Hsp70 expression between the two groups (Fig. 1). One explanation for this result may be that foragers have hotter thoraces, but not heads, than hive bees. While this is true in some circumstances (Stabentheiner, 2001), it is also possible that elevated Hsp70 expression in forager thoraces may be due to the extreme protein degradation, repair, maturation and replacement needed by the heavily taxed forager flight muscles (conservatively estimated to contract over 4 million times per day based on 5 h of flight per day and 240 wingbeats per second; Winston, 1987; Harrison et al., 1996a).

The development of flight and metabolic capacity in honey bees is also subject to circulating juvenile hormone (JH) levels, which rise before the onset of foraging (Elekonich et al., 2001; Jassim et al., 2000) and typically are much higher in foragers compared with nurses (reviewed by Bloch et al., 2002). Honey bees that have had their corpora allata (the sole source of JH) surgically removed still become foragers, but at an older age than intact bees. Treatment with the JH analog methoprene after allatectomy eliminates this delay (Sullivan et al., 2000). Hence, JH does not activate foraging (Elekonich and Robinson, 2000) but rather influences the pace at which honey bees develop into foragers. Mortality during the first orientation flight of foragers is higher in allatectomized honey bees than in sham and untreated honey bees (Sullivan et al., 2000, 2003). Furthermore, allatectomized honey bees have significantly reduced ground speeds during orientation flights, decreased flight ability and lower flight metabolic rates relative to sham and untreated honey bees (Sullivan et al., 2003). Endocrine influences on muscle development and capacity are well known in both insects and vertebrates. For humans and other mammals, the effects of testosterone and other steroids to increase muscle mass, increase power output, decrease protein degradation and increase amino acid utilization in mammals are well known (Herbst and Bhasin, 2004; Bhasin et al., 2001). Thyroid hormone, the most similar to JH in structure, acts on muscle function at the cellular level by modulating sodium, potassium and calcium ATPase levels (Everts, 1996). In a variety of other insects, JH, ecdysone and octopamine are all known to influence muscle development, flight ability and life history transitions (Applebaum and Heifetz, 1999; Dingle and Winchell, 1977; Pener and Yerushalmi, 1998; Rankin, 1991; Roy and VijayRaghavan, 1999). The wing polymorphic crickets (*Gryllus firmus*) are particularly well studied with regard to reproduction, lipid biosynthesis, flight capability and endocrine control. Adult JH titers induce ovarian development and, in contrast to the honey bee, increased JH in early adulthood induces flight muscle histolysis in the flight-capable morphs (Zera and Cisper, 2001; Zhao and Zera, 2002, 2004).

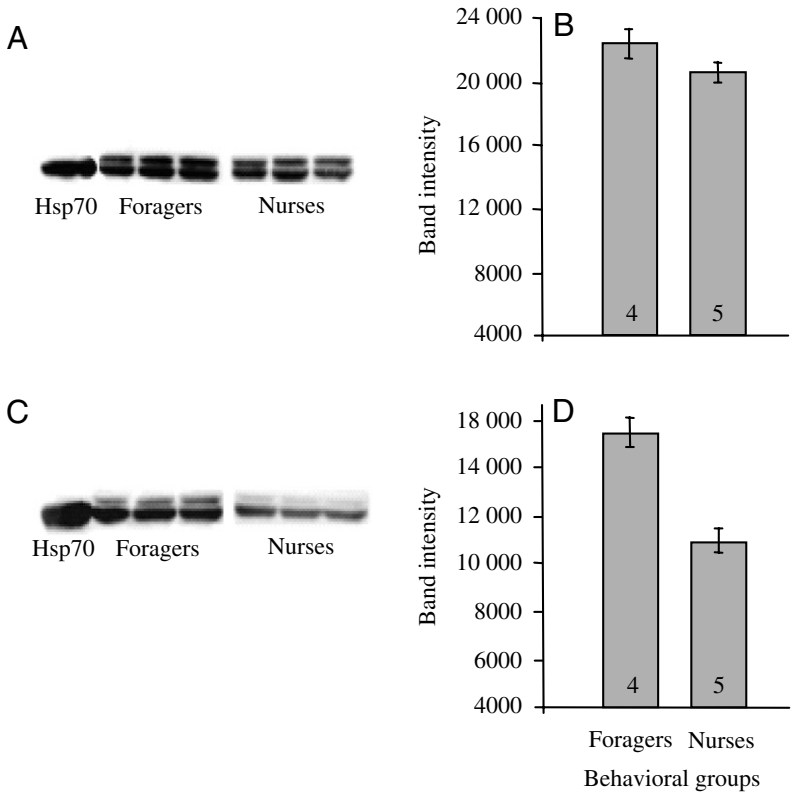

Fig. 1. Hsp70 expression in honey bee nurses and foragers. (A,B) Hsp70 expression in heads; (C,D) Hsp70 expression in thoraces. Hsp70 family proteins were isolated on western blots labeled with a Hsp70 mouse monoclonal antibody (Sigma, St Louis, MO, USA; H5147) and an anti-mouse secondary antibody conjugated to horseradish peroxidase (Sigma). Proteins were visualized with enhanced chemiluminescence and quantified on a Typhoon Phosphorimager (Amersham, UK). The Hsp70 standard is 0.5 μg of purified Hsp70 protein (from bovine brain; Sigma; H9776). Numbers in bars indicate sample size.

The examples discussed here represent but few of the numerous possible molecular and biochemical changes that underlie the maturation of honey bee flight muscle performance. With the recent completion of the honey bee genome sequence, research is underway to (1) identify

additional molecular and biochemical variation associated with the transition to foraging, (2) determine whether such variation corresponds to the development of aerodynamic and metabolic performance and (3) determine whether such variation is driven primarily by age or behavior.

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