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# QUEEN AND WORKER INFLUENCE ON SEX ALLOCATION PATTERNS IN THE HONEYBEE, APIS MELLIFERA

By

Katie Elizabeth Wharton

# A DISSERTATION

Submitted to Michigan State University in partial fulfillment of the requirements for the degree of

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Department of Zoology Program in Ecology, Evolutionary Biology, and Behavior

#### ABSTRACT

#### QUEEN AND WORKER INFLUENCE ON SEX ALLOCATION PATTERNS IN THE HONEYBEE, APIS MELLIFERA

By

#### Katie Elizabeth Wharton

Since Darwin, biologists have sought to understand the ways in which natural selection shapes the ability of organisms to adjust their investment in the sexes. A social insect colony, comprised of many individuals, faces the challenge of coordinating its individuals to achieve an adaptive allocation to the sexes. In this dissertation, I focus on the ways in which sex allocation in honeybee colonies is shaped by the actions and interactions of the queen and workers.

Previously, attempts to understand how colony members shape colony sex allocation patterns have generally focused on social insect species in which the queen and workers favor different colony sex allocation optima. This queen-worker conflict over sex allocation is expected to be minimal or absent in species with a highly polyandrous queen, including the honeybee. In these species, understanding how the queen and workers influence colony sex allocation might provide important insights into how colonies efficiently and flexibly respond to environmental conditions. To better understand how queens and workers shape colony sex allocation patterns when conflict is expected to be minimal or absent, I conducted experiments using the honeybee.

The queen lays nearly all of the eggs in her colony and might therefore play a key role in her colony's investment in males (drones). To test whether the queen's egg-laying decisions impact colony sex allocation patterns, I manipulated the previous egg-laying experiences of queens and examined their subsequent tendency to lay worker or drone eggs. My results indicate that the queen has the ability to regulate her production of drone eggs, and in turn has the ability to influence her colony's regulation of investment in male reproductive function. More generally, this work reveals that the honeybee queen and workers share control over their colony's sex allocation.

After the queen lays eggs, the workers rear the eggs to adulthood. However, workers occasionally eliminate a portion of their colony's immature males. To better understand how brood rearing decisions by workers impact colony patterns of sex allocation, I manipulated the abundance of older male brood in colonies and quantified the tendency of workers to rear young larvae. Workers eliminated a greater proportion of young male larvae when I increased the abundance of older male brood than when I decreased it. While earlier studies suggest that male elimination reflects conflict between the queen and workers, my results raise the possibility that male elimination might sometimes reflect adaptive adjustment of male reproductive function, potentially increasing colony efficiency in the interests of all colony members.

Historically, biologists have primarily investigated sex allocation theory through the study of offspring sex ratios. I suggest that our understanding of sex allocation will be aided by applying analyses that use the raw numbers of males and females, rather than the ratio of males to females, as the primary variables of interest. To illustrate this point, I examine existing datasets of offspring production and demonstrate that analyzing sex allocation behavior can lead to fundamentally different biological conclusions than those drawn from analyzing sex ratio behavior.

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# CHAPTER 1 INTRODUCTION

#### **1. General Introduction**

Since Darwin (1874), biologists have sought to understand the ways in which natural selection shapes the ability of organisms to adjust their investment in the sexes. An organism's allocation of resources to male and female reproductive function is termed its sex allocation (Charnov 1982). In the field of behavioral ecology, sex allocation theory has led to major advances in our understanding of how organisms perceive and respond to changing environmental conditions. In this dissertation, I focus on how sex allocation in honeybee (*Apis mellifera*) colonies is shaped by the decisions of the queen and workers.

Social insect colonies, like many individual organisms, must allocate resources appropriately between growth and reproduction. Honeybee colonies reproduce in two distinct ways: by swarming, a fission process in which the queen and over half of the worker bees leave to establish a new colony, and by producing and maintaining males (hereafter drones), whose sole apparent function is to mate with virgin queens in the population. This latter "male" mode of reproduction allows a colony to introduce the genes shared by its members into other colonies in the population, and is therefore an important route by which colony members increase their inclusive fitness.

Honeybee colonies actively regulate their investment in male reproductive function. Drones typically compose 5-10% of the adult population in a honeybee colony (Seeley and Morse 1976; Page and Metcalf 1984). The colony, however, is able to adjust

its investment in drones and workers in accordance with environmental factors (Page and Metcalf 1984). How colonies manage to do this through the actions and interactions of the queen and workers presents an open and interesting question in social insect biology (Bourke and Franks 1995), and is the focus of this chapter.

The queen lays nearly all of the eggs in her colony and is therefore an important participant in her colony's brood production process. In honeybees and other haplodiploid species, haploid (unfertilized) eggs develop into males and diploid (fertilized) eggs develop into females. The queen therefore has the opportunity to influence her colony's sex allocation by controlling whether or not she fertilizes her eggs (Ratnieks and Keller 1998). Despite this knowledge, biologists have long assumed that worker honeybees control their colony's sex allocation. In Chapter 2, I inquire whether the honeybee queen has the ability to influence her colony's investment in male reproductive function.

The workers, or non-reproductive females in the colony, perform many essential tasks for their colony's brood production process. For example, workers build the wax cells that are used to rear the brood, clean the cells in preparation for deposition of an egg, and provide food to the developing larvae. Occasionally, however, workers will cannibalize developing larvae and therefore fail to rear all of the queen's eggs to adulthood (Woyke 1977). By facultatively rearing workers or drones, workers might adaptively adjust their colony's sex allocation in response to changing environmental conditions. In Chapter 3, I investigate how a honeybee colony's investment in male reproductive function is shaped by the brood rearing decisions of the workers.

Historically, biologists have primarily investigated sex allocation theory through

the study of offspring sex ratios. In Chapter 4, I suggest that biologists might gain more insightful interpretations of their system of study by treating the raw number of males and number of females (sex allocation), rather than the ratio of males to females (sex ratio), as the variables of interest in their studies. To illustrate this point, I compare the results of taking a sex allocation versus a sex ratio approach to datasets documenting offspring production by honeybee queens (Wharton et al. 2007) and female parasitoid wasps (Shuker et al. 2004).

In the remainder of this chapter, I review the literature on how honeybee colonies adjust their investment in male reproductive function, and I highlight promising areas of future research within this topic. I begin by identifying five environmental conditions and explaining how each affects colony-level patterns of drone rearing. Next, I define the key stages involved in drone production and maintenance and focus on how drone rearing at each stage changes in relation to the five environmental conditions. I conclude by emphasizing the importance of studies that examine how colony sex allocation is shaped by the actions and interactions of the queen and workers.

# 2. Colony-level patterns of drone production based on environmental factors

Honeybee colonies regulate their production and maintenance of drones in accordance with several environmental factors. In this section I identify five of these factors and discuss colony patterns of drone rearing in relation to each.

# 2.1 Season

The number of drones present in a honeybee colony varies over the course of a year. A colony's drone population increases during the spring and reaches a peak in the late spring or early summer. Although this peak in drone production also coincides with the peak in worker production, it is during this time of year that colonies produce the highest proportion of drone brood (Free and Williams 1975). This peak occurs just prior to the population's main swarming season, when virgin queens are most abundant (Allen 1958; Allen 1965b; Page 1981; Lee and Winston 1987). As a result, colonies have the greatest number of drones during the time of year when drones are most likely to successfully mate. The colony's drone population slowly decreases throughout the late summer and into the fall, and typically dwindles to zero by winter.

The seasonal factors that influence these trends in drone production are unknown. While colonies might adjust drone production in relation to season itself (day length and temperature), the trends more likely arise from a combination of season and other environmental cues (colony size and availability of food) that vary with season.

#### 2.2 Size of colony

Honeybee colonies refrain from producing drones until the colony has a sufficiently large population of adult workers. The population of adult workers in a colony is naturally low following a swarming event, and swarms that establish new colonies prevent early drone production by not constructing drone comb (the wax cells in which drones are reared) until an average of 22 days after establishment (Lee and

Winston 1985). Once established, larger swarms build a greater proportion of drone cells more quickly than do smaller swarms, indicating a strong effect of swarm size on drone comb investment (Henderson 1991). In addition, small swarms that are composed of fewer than 10,000 workers sometimes build little or no drone comb at all, indicating that small colonies limit their investment in drones until they've grown larger (Lee and Winston 1985).

As small colonies increase their production of brood, they also increase the proportion of drone brood (Free and Williams 1975). However, once colonies reach a large size (more than 12,000 adult workers), there doesn't appear to be any relationship between the number of workers reared and the percentage of drone brood in the colony (Page and Metcalf 1984). Colonies therefore reach an upper limit in their investment in drones.

#### 2.3 Food availability

Drones are more costly to produce and maintain than workers (Seeley 2002; Hrassnigg and Crailsheim 2005), so colonies should regulate drone production in accordance with the availability of food. Seeley and Mikheyev (2003) confirmed this in a controlled experiment. They maintained a group of colonies in a food-plentiful environment and observed that the colonies produced similar amounts of drone brood. They then transferred the colonies to an island with very few food sources and experimentally manipulated food availability by provisioning half of the colonies with sugar water and leaving the other colonies to rely on only the island's limited food

sources. On the island, the food-supplemented colonies continued to produce many drones, but the non-supplemented colonies lowered their production of drones.

Honeybee colonies also decrease their production of workers when foraging conditions are poor (Schmickl and Crailsheim 2001; Schmickl and Crailsheim 2002), but it is unknown how honeybee colonies jointly regulate drone and worker production during periods of low food availability. Since the value of drones changes in relation to workers throughout the year, colonies might be expected display seasonal trends in how they jointly produce drones and workers in response to food availability, but this has not yet been tested.

# 2.4 Amount of drone brood and number of drones already present in the colony

Colonies adjust their production of immature drones (hereafter drone brood) in accordance with the amount of drone brood already present. Specifically, when drone brood is added to colonies, the colonies lower their production of additional drones (Free and Williams 1975). This demonstrates that that the presence of drone brood in a colony inhibits the colony's production of additional drones. Drone production is therefore regulated by a negative feedback process.

Studies that tested whether colonies adjust their production of drones in accordance with the number of adult drones present have produced mixed results. Rinderer et al. (1985) added adult drones to colonies and found that the colonies subsequently lowered their drone production. However, Henderson (1994) removed drones from colonies and found that colonies did not increase drone production. These

mixed results call into question whether, and if so how, honeybee colonies can assess the size of their population of adult drones.

Future work might better examine the trade-offs that colonies face by investing in drones. Drone rearing does not appear to trade off with worker rearing, because colonies that are prevented from investing in drones do not produce more workers than colonies allowed to rear drones (Allen 1963; Allen 1965a; Page and Metcalf 1984). Drone rearing might trade off with honey yields, an important means by which colonies survive the winter, but there is both supporting and contradictory evidence that drone rearing affects honey yields (Allen 1965a; Johansson and Johansson 1971; Seeley 2002). These results raise questions about the life history trade-offs that honeybee colonies face when investing in drones.

#### 2.5 Queen presence/absence

The environmental factors discussed above have only been evaluated in colonies where the queen is present, termed queenright colonies. In queenright colonies, the queen produces approximately 99.9% of the colony's adult males (Visscher 1989). If the queen becomes inviable or dies, the colony will attempt to rear a replacement queen. If that attempt fails and the colony becomes "hopelessly" queenless, drones become the sole source through which the colony can pass along genes. Accordingly, the colony increases its investment in drones relative to workers. This happens naturally because some workers reared in queenless colonies develop functional ovaries and are able to lay drone (unfertilized) but not female (fertilized) eggs.

In queenless colonies, workers begin laying eggs around 24 days after the queen's disappearance, and their production of drone eggs is short and synchronous (Page and Erickson 1988). Which workers are most successful in laying eggs is difficult to infer, because workers consume or "police" many of the eggs (Miller and Ratnieks 2001). Additionally, subfamilies of workers vary in their tendencies to produce and consume eggs (Robinson et al. 1990), and younger workers appear to be more successful at laying eggs than older workers (Delaplane and Harbo 1987). Despite the oophagy that occurs, worker egg-laying can result in 6000 additional adult drones for a queenless colony (Page and Erickson 1988). However, because the workers cannot produce new workers or a new queen, the colony cannot maintain these drones for long before the colony dies.

# 3. Key stages in drone production and maintenance

Colonies produce and maintain drones in a multi-staged sequential process (Figure 1.1). At each stage, either the workers or the queen have the opportunity to shape the colony's investment in drones. In this section I examine each of these stages in detail. For each stage, I present a brief overview of the stage's importance for colony drone production, review how the bees modify the colony's investment in drones in response to the environmental factors listed in the previous section, and highlight aspects of the stage that aren't well understood. A summary of this section is provided in Table 1.1.



Figure 1.1. Drone production and maintenance in a honeybee colony: a sequence of actions taken by the workers and the queen. At each step, the bees have an opportunity to alter the colony's investment in drones in response to environmental conditions. Arrows indicate typical sequences of events. Drone cells are larger than worker cells, and drones are normally reared in these larger cells (I). The worker and drone cells are used either for food storage (nectar and pollen; darker cells in diagram) or are cleaned in preparation for rearing brood (II). The queen typically lays unfertilized (drone) eggs into drone cells, and fertilized (female) eggs into worker cells (III). Workers then tend the larvae, but don't necessarily raise all of the larvae to adulthood (IV). Adult drones remain colony members until they mate with a virgin queen in the population, die, or are evicted by workers (V). See sections 3.1 - 3.5 of this chapter for details of each stage.

Table 1.1. Current understanding of how colonies adjust drone production in relation to environmental conditions (rows) for each of the stages involved in drone production (columns). D = drone; W = worker. See sections 3.1 - 3.5 of this chapter for additional details.

	Workers	Workers clean	Queen lays	Workers	Workers
	build comb	cells/fill cells	eggs	rear/fail to	maintain/evict
		with nectar		rear larvae	adult drones
Season	D comb	D cells are used	D egg	Survival of D	D eviction is
	construction is	primarily for	production is	larvae is	higher in the
	highest in the	brood rearing in	higher in	variable	fall than in
	spring and	spring/summer	spring-early	across	spring or
	early summer	and primarily	summer than	season;	summer
	_	for nectar in	in late	survival is	
		fall	summer-fall	lowest in the	
				fall	
Size	Larger	Unknown	Unknown	Unknown	Unknown
	colonies build		how colony		
	more D comb,		size affects D		
	and build it		egg		
	more quickly,		production		
	than smaller				
	colonies				
Food availability	Colonies must	Unknown	Egg	Fewer brood	D eviction is
	have nectar		production is	survive under	higher in unfed
	intake to build		lower during	poor foraging	colonies than in
	any comb at		poor food	conditions;	fed colonies
	all; unknown		conditions;	relative	
	how food		unknown how	survivals of D	
	affects		this affects	and W brood	
	proportion of		relative	are unknown	
	D comb built		production of		
			D and W eggs		
Amount of drone	D comb alone	Unknown	D brood	Survival of D	Unknown
brood and adult	inhibits D		inhibits D egg	larvae is	
drones present	comb		production;	lower when	
in colony	construction;		unknown if	colonies	
	D brood		adult D	contain D	
	enhances this		inhibit D egg	brood;	
	inhibition.		production	unknown	
	Unknown if			how survival	
	adult D inhibit			of D larvae is	
	D comb			influenced by	
	construction			adult D	
Queen	Queenless	Unknown	If queen is	Survival of	D eviction
presence/absence	colonies build		absent,	queen-laid D	higher in
	less comb		workers lay D	brood	queenright
	overall, but a		but not W	increases in	colonies than in
	greater		eggs	queenless	queenless
	proportion of			colonies; low	colonies
	the comb built			survival of	
	is D comb			worker-laid D	
				brood	

#### 3.1 Workers build comb

Workers build the wax cells that are used by the colony to store food and rear bees. These cells are either small in size (worker cells) or larger in size (drone cells). Drone cells typically constitute 15-20% of the total comb area (Seeley and Morse 1976). Though bees occasionally reshape one type of cell into the other type under unnatural conditions (see Free and Williams 1975), they typically don't alter the wax comb that has already been built. Since drones cannot be reared properly in the smaller worker cells, the number of drone cells in a colony limits the number of drones that can be reared at any one time. However, if colonies are provisioned with an excessive amount of drone comb, they won't use all of it for rearing drones, indicating that a colony's regulation of drones is determined by more factors than just the amount of comb in the colony (Allen 1965a). Of all of the stages involved in drone production, the construction of comb has been the most extensively studied. For an extensive review of the factors that govern comb construction in honeybees, see Pratt (2004).

Workers build drone comb mostly in the spring and early summer, indicating that season influences drone comb construction (Free 1967). Two lines of evidence support this assertion. First, swarms established earlier in the year produce more drone cells than swarms (of an identical size) established later in the year (Lee and Winston 1985). Second, colonies build the highest proportion of drone cells in May through July (Free and Williams 1975). This occurs despite the fact that the authors in that study controlled for both food availability and drone brood presence by supplementing their colonies with sugar syrup throughout the year, and by removing built drone comb on a weekly basis.

These results therefore are consistent with the idea that season itself (day length and temperature) influences drone comb construction.

Drone comb construction is influenced by colony size. Small colonies construct fewer drone cells than larger colonies (Free 1967; Lee and Winston 1985; Henderson 1991). This pattern holds for established swarms as well: small swarms usually build only a very small proportion of drone comb at first (Taber and Owens 1970; Henderson 1991), whereas larger swarms will build drone comb more immediately (Free and Williams 1975; Henderson 1991). Smaller colonies likely suppress their construction of drone comb in order to allow the colony to grow large enough to support a population of drones.

Drone comb construction is regulated by negative feedback. Workers reduce their construction of drone comb when drone comb is already present (Free 1967; Free and Williams 1975; Pratt 1998), and they further reduce drone comb construction if drone brood is present as well (Pratt 1998). Based on these trends, one might expect that workers reduce drone comb construction when the colony contains an abundance of adult drones, but this remains an open question.

Drone comb construction is influenced by whether or not the colony has a queen. When the colony is queenless but workers are rearing queens, the workers build a higher proportion of drone cells (Free 1967), but less comb overall, than do queenright colonies (Pratt 2004). This is not because the queen constructs comb or directs comb construction (Pratt 1998), but presumably occurs because selection has acted on queenless colonies to invest more in drones, their sole method for passing on genes. In queenright colonies, food must be available in order for workers to construct any comb at all (Pratt 2004), but

how the amount of food available influences the colony's relative investment in drone cells to worker cells is largely unknown. One study found that colonies did not alter drone comb construction depending on food availability, but that study used too few colonies to make a statistical inference (Sasaki and Obara 2001).

#### 3.2 Workers clean cells or fill cells with nectar

Worker bees perform the actions of cleaning the brood cells and storing or removing food from the cells. When the cells have been cleaned of debris and do not contain any pollen or nectar, they are available to receive an egg from the queen (Winston 1987). Since the queen lays eggs continuously and workers clean and use cells constantly, whether a particular cell is used for food storage or for rearing brood depends upon a dynamic interplay of actions taken by both the queen and the workers.

The use of drone and worker cells for egg deposition appears to be independent of the use of these cells for either cleaning or food storage. In one study, the decision of the queen to deposit an egg in a drone or worker cell was not constrained by the decisions of workers to fill drone and worker cells with nectar (Wharton et al. 2007). In another study, workers were equally likely to clean either worker or drone cells (Sasaki et al. 1996). Although the decisions of the queen and workers might be independent in some circumstances, they are likely dependent when a colony has limited cells available for brood or food. Future work might address whether in such cases the workers primarily limit the cells available to receive eggs, or whether the queen primarily limits the cells available for food storage. Whether workers respond to environmental factors for cell cleaning activities or for storing food remains largely an open question. The use of drone comb for these activities appears to depend on season: drone comb contains mostly drone brood in the early summer and mostly food in the early fall (Free and Williams 1975; T.D. Seeley, personal communication). This seasonal pattern might arise because workers refrain from filling drone cells with food until later in the season, because queens reduce the number of eggs they lay into drone cells later in the season, or because of some combination of these two factors. To date, few studies have examined how the use of a cell depends on colony size, food availability, the presence of drones, or the colony's queen status. This paucity of research likely stems from the difficulty of determining how patterns of brood and food deposition arise from a complex interplay between the decisions of the workers and the queen.

# 3.3 Queen lays eggs

The queen honeybee contributes to colony drone production by laying drone (unfertilized) eggs into available drone cells. She deposits worker (fertilized) eggs into worker cells. The queen sometimes lays drone and worker eggs in a ratio that deviates from the ratio of drone and worker cells (Henderson 1991; Wharton et al. 2007), suggesting that the queen can shape brood production patterns by choosing whether to lay eggs in either drone or worker comb. These eggs eventually develop into larvae, which might be consumed by the workers (see section 3.4). Therefore, to understand how the queen adjusts her drone egg production in response to environmental conditions, it is

necessary to examine the colony's egg (rather than larvae or pupae) sex allocation. Few studies focus on the egg sex allocation patterns within a colony, and we address potential reasons for this lack of research in section 4. Nevertheless, a few studies have examined queen egg-laying decisions, and I outline those results here.

A colony's number of drone eggs depends on the season. The proportion of drone eggs is higher in the spring and summer than in the fall (Free and Williams 1975), though this trend might be due to higher food availability in the summer. Sasaki and Obara (2001) examined the interaction of season and food availability on the queen's egg laying decisions. They found that when colonies are continuously supplied with food throughout the year, queens lay more drone eggs in the spring and summer than in the fall. Additionally, while the queen lays few drone eggs in the fall independent of food condition, in the late spring she appears to produce a higher proportion of male eggs during good food conditions than during poor food conditions. However, that study used a within-subjects design and did not control for the order of food condition treatments. Therefore, how the queen alters her production of drone eggs in response to food availability should be tested in future studies. Additionally, future studies might examine whether the queen's production of drone eggs changes in response to colony size.

The honeybee queen's production of drone eggs is regulated by negative feedback. Studies concluding this had found that queens who had recently laid drone eggs subsequently produced fewer drone eggs than queens who had recently laid only worker eggs (Sasaki et al. 1996; Wharton et al. 2007). In the study by Wharton et al. (2007), the authors followed the eggs to the pupal stage and found that the queen's egglaying decisions influenced colony-level drone production patterns. Additionally, the

authors controlled for season, colony size, and food availability, and found that the decisions of the queens were not constrained by worker decisions to fill cells with nectar. That study therefore demonstrated that the queen altered her egg-laying patterns by detecting and responding to either her own previous egg-laying experience or the presence of drone brood in the colony. Which of these two factors was a greater determinant of queen egg-laying patterns is an attractive topic for future study.

Overall, how queens alter drone production in response to environmental factors is an area that has been largely overlooked. We discuss reasons for why this might be in section 4 of this chapter.

#### 3.4 Workers rear/fail to rear larvae

Workers sometimes consume a portion of their colony's developing larvae (Woyke 1977). Workers presumably eliminate larvae when environmental conditions are unfavorable for rearing brood. These actions might serve to recycle colony resources before the larvae become too expensive for the colony to support (Webster et al. 1987). Consistent with this idea, workers selectively destroy younger larvae, which would cost more to rear to maturity than older larvae (Woyke 1977; Schmickl and Crailsheim 2001; Schmickl and Crailsheim 2002; Schmickl et al. 2003). Additionally, although workers rarely consume pupae, they preferentially consume younger rather than older pupae (Newton and Michl 1974).

The tendency of workers to eliminate males fluctuates over the season and with the colony's queen status. During the spring and fall, workers consume more drone

brood than worker brood, but in the summer the survivals of drone and worker brood are low and approximately equal (Fukuda and Ohtani 1977; Woyke 1977). These results suggest that workers consume more drone larvae in the fall when drones are of little value to the colony. In queenless colonies, the survival of queen-laid drone and worker larvae increases (Woyke 1977), although once workers begin to lay eggs, many workerlaid drone eggs are destroyed (Miller and Ratnieks 2001).

The tendency of workers to eliminate males is influenced by the amount of drone brood in the colony. This was confirmed in a recent study by Wharton et al. (in review). They manipulated the abundance of older male brood in colonies and found that the survival of younger male larvae was lower when the abundance was increased than when it was decreased. Meanwhile, the survival of worker larvae was high across colony conditions. These results suggest that the brood rearing decisions of workers assist the colony in regulating its investment in male reproductive function. This study is one of only a few to date that directly compares the survival of drone and worker larvae in an experimental manipulation.

The tendency of workers to eliminate males likely is influenced by colony food conditions, but how food conditions affect the relative rates of cannibalism for drone and worker larvae awaits future study. The tendency of workers to consume worker larvae increases during periods of poor food availability (Schmickl and Crailsheim 2001). Interestingly, under poor colony food conditions, the workers preferentially consumed younger (less than 3 days old) rather than older (4-5 day) larvae and also preferentially withheld nursing from the younger larvae (Schmickl and Crailsheim 2001; Schmickl and Crailsheim 2002; Schmickl et al. 2003). The colonies in these studies contained only

worker brood, so it is unknown how the workers would have treated drone larvae under similar conditions.

In general, the circumstances under which workers cannibalize larvae are poorly understood. This is largely because very few studies to date compare the survival of drone and worker larvae in a manipulative experiment. An exception to this is the study mentioned above by Wharton et al. (in review). I suggest that we need more manipulative studies to determine whether and under what conditions workers selectively destroy drone or worker larvae. To selectively destroy either drone or worker larvae, workers would need to distinguish between the two. While it is unknown if workers can distinguish between drone and worker larvae based on cell size alone, workers are likely not able to distinguish between the two sexes based on chemical cues until the larvae are 3-4 days old, which is approximately seven days after the queen lays eggs (Sasaki et al. 2004). Much more work remains to be done in this area.

# 3.5 Workers maintain or evict drones

Besides producing drones, colonies maintain adult drones. Adult drones are costly for the colony to maintain because a drone consumes approximately four times the amount of food that a worker consumes (Winston 1987). Colonies will maintain a drone until one of the following happens: the drone mates successfully with a virgin queen (drones die upon mating), the drone dies, or workers evict the drone from the colony in a process known as "drone eviction." During drone eviction, workers force drones out of the colony and prevent them from reentering (Free 1957). Since the evicted drones cannot access the colony's food stores, they soon die of starvation. A drone's age appears to affect his likelihood of being evicted; Free (1957) observed that workers selectively harassed older drones while permitting younger drones to remain. Drone eviction takes place gradually over the autumn and is most likely to occur on warmer days when the workers are more active in the colony (Morse et al. 1967). Although the exact cues that trigger drone eviction are unknown, it is thought that workers evict drones when the expected cost of maintaining drones outweighs the expected fitness benefit.

Since the fitness benefit of keeping and maintaining drones fluctuates with environmental conditions, colonies exhibit greater levels of drone eviction under particular environmental circumstances. Workers evict more drones in the fall than in the spring (Morse et al. 1967); drone eviction before the fall rarely occurs. During the fall, queenright colonies evict more drones during periods of poor food availability (Free and Williams 1975). Additionally, when foraging conditions are poor in the fall, queenright colonies evict more drones than do queenless colonies; when foraging conditions are good, the colony's queen status does not affect the amount of drone eviction (Free and Williams 1975).

There is still much to learn about drone eviction. In particular, it is unknown how colony size (or a reduction in colony size following a swarm event) affects drone eviction. Observations support the idea that colony size might play a role: when frames of brood and bees were brought into a small (2-frame) observation hive during midsummer, workers soon evicted the drones (Wharton, personal observation), although this

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doesn't always happen (Free 1957). Additionally, future research should better elucidate the proximate cues that trigger the workers to recognize that their colony has an excess number of drones.

#### 4. Conclusions

Drones are an important but costly investment for the honeybee colony, so colonies regulate the number of drones they produce. This regulation of drone production arises from the actions and interactions of the queen and workers, who must respond to environmental factors that affect the expected fitness value of rearing drones. To date, research with honeybees has elucidated the responses of colony members to several environmental influences. However, this work is by no means exhaustive, and has only begun to shape our understanding of honeybee brood production dynamics and colony organization. Future work in this area is crucial in order to better understand how the individuals in social insect colonies coordinate their actions to achieve adaptive colony decisions.

The emerging picture of brood production in honeybees is that the queen and workers share control over their colony's sex allocation. Historically, the queen was viewed as an egg-laying "machine" that did not regulate colony-level patterns of sex allocation. Recent evidence suggests that this previous view is incorrect; the queen's egg-laying decisions can shape her colony's regulation of investment in male reproductive function (see section 3.3). Additionally, workers play an important role in colony sex allocation patterns by building and preparing wax cells to hold the brood,

rearing or destroying the larvae, and maintaining or evict adult drones (sections 3.1, 3.2, 3.4, 3.5). Together, the decisions of the queen and workers provide a colony with many stages and strategies by which it might adjust sex allocation in response to environmental conditions.

In many social insect species, the queen and workers are expected to favor different sex allocation optima (Free 1957; Hamilton 1964; Trivers and Hare 1976; Strassmann 2001). This queen-worker conflict over sex allocation arises because of the haplodiploid system of sex determination, by which workers are typically 3 times more related to their sisters than to their brothers whereas the queen is equally related to her daughters and sons. In species characterized by queen-worker conflict, the extent to which colony members can influence sex allocation provides insights into the distribution of power within the colony, where power is defined as the ability to do or act in situations of conflict (Beekman et al. 2003; Beekman and Ratnieks 2003).

In species in which the queen mates with many males (as in the honeybee), queen-worker conflict over sex allocation is theoretically very small or absent (Moritz 1985; Ratnieks et al. 2006). Species that exhibit an extremely high queen mating frequency include the honeybee (Neumann and Moritz 2000), the Florida harvester ant *Pogonomyrmex badius* (Rheindt et al. 2004), and the African army ant *Dorylus anomma* (Kronauer et al. 2004). In these species, queens and workers theoretically "agree" on their colony's sex allocation. In species characterized by an absence of queen-worker conflict, the extent to which colony members can influence sex allocation provides important insights into how colonies efficiently and flexibly respond to environmental conditions.
Whether or not there is conflict between the queen and workers over colony sex allocation, the study of queen and worker influence on colony sex allocation patterns has great potential to advance our understanding of the dynamics and organization of social insect colonies. Increasingly, researchers are recognizing social insects as an excellent model system for addressing questions about how living systems organize their individual components in order to cope with a dynamic environment (Camazine et al. 2001). Research that elucidates the effects of queen and worker decisions on colony sex allocation will therefore broaden our understanding of how social insect colonies, and in turn other complex living systems, coordinate their group members in order to cope with environmental uncertainty. This research is also likely to provide key insights into the behavioral ecology of social insects, and more broadly, the evolution of cooperation.

# CHAPTER 2 THE HONEYBEE QUEEN INFLUENCES THE REGULATION OF COLONY DRONE PRODUCTION

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#### Introduction

Social insect colonies, like many individual organisms, must allocate resources appropriately between growth and reproduction. How colonies manage to do this is not well understood, because little is known about how colony life history decisions are governed by the actions and interactions of colony members (Bourke and Franks 1995). Recent work has begun to focus on how one colony-level reproductive decision, the investment in males, is influenced by the queen and the workers (Herbers et al. 2001; Beekman et al. 2003; Beekman and Ratnieks 2003; Mehdiabadi et al. 2003; Pen and Taylor 2005; Ratnieks et al. 2006). However, the attempt to understand how the queen and workers cooperate when allocating colony resources to reproduction is complicated by the fact that their actions may instead spring from conflict.

A colony produces males through the actions of the queen and the workers, who are potentially in conflict over their colony's sex allocation, or investment in males versus females (Ratnieks et al. 2006). While there are other types of queen-worker conflict (see Ratnieks et al. 2006), we consider only conflict over sex allocation for this paper. The potential for this conflict is due to the haplodiploid system of sex determination, whereby the queen is equally related to her sons and daughters whereas workers are on average more closely related to their sisters than to their brothers (Hamilton 1964). When colonies have one queen that is singly mated, workers are three times more related to their sisters than to their brothers, leading to the prediction that the queen and workers are in conflict over their colony's sex allocation and that each party should attempt to bias sex allocation in its favor (Trivers and Hare 1976). One way in which workers might do so is by selectively eliminating male larvae, and workers of

several species of ants apparently do this (Aron et al. 1994; Aron et al. 1995; Keller et al. 1996a; Keller et al. 1996b; Sundström et al. 1996). Given that workers in some species appear to control the colony's investment in males, whether queens can influence male investment by modulating their egg sex ratio has recently gained interest (Aron et al. 1995; Keller et al. 1996b; Cremer and Heinze 2002; Beekman and Ratnieks 2003; Mehdiabadi et al. 2003; de Menten et al. 2005a; de Menten et al. 2005b; Koedam et al. 2005).

Although the queen and workers might experience conflict, their inclusive fitness is determined largely by colony success, so they share an interest in adaptively regulating the colony's investment in reproduction. So, while the ability of the queen or the workers to influence their colony's investment in males is consistent with the hypothesis that they are in conflict, an alternative explanation is that queens and workers are assessing local information about the value of rearing males and are cooperatively but sequentially shaping the colony's allocation to reproduction. For example, the queen might modulate her egg sex ratio to ensure that the colony is well-supplied in males, and workers might eliminate males later if conditions become unfavorable for raising sexuals. We term this hypothesis queen-worker cooperation. Because queens and workers might agree over their colony's allocation to reproduction but also experience conflict over sex allocation (Herbers et al. 2001), queen-worker conflict and queen-worker cooperation are not mutually exclusive scenarios. Any ability of queens or workers to influence their colony's male investment may therefore reflect a strategy of biasing sex allocation in their favor (conflict), a strategy of obtaining the colony's adaptive allocation to reproduction (cooperation), or some mixture of both. For this reason, it is difficult to

interpret the functional significance of queen and worker sex-biasing abilities when there is a high potential for queen-worker conflict.

While cooperation between queens and workers is likely to be entangled with conflict in many species, highly polyandrous species should have little potential for conflict over sex allocation (Ratnieks et al. 2006). This is because when the queen mates with many males, as in honeybees (Estoup et al. 1994; Neumann and Moritz 2000), African army ants (Kronauer et al. 2004), and harvester ants (Rheindt et al. 2004), the average relatedness between workers is low so workers are almost equally related to their brothers and sisters. As a result, the queen and workers should favor a nearly equal investment in the sexes (Moritz 1985; Ratnieks et al. 2006). Therefore, highly polyandrous species provide an opportunity to study whether queens and workers have evolved the ability to influence the colony's investment in males when conflict over sex allocation is not in the picture. In this paper, we investigate one such species, the honeybee, and ask whether the queen has the ability to influence colony patterns of drone (male) production.

The size of the drone population in a honeybee colony depends upon a sequence of actions taken by the queen and workers (Figure 2.1). With each action, there is an opportunity for the bees to alter the investment in drones. Workers construct the wax combs in which brood are reared, and thus control the colony's investment in drone and worker cells (Pratt 2004). Drone cells are larger than worker cells and allow drones to develop to full size; therefore, the number of drone cells provides an upper limit for the number of drones that can be reared at any one time. Additionally, workers use some cells for the storage of pollen and nectar, and therefore determine the availability of



Figure 2.1. Drone production and maintenance in a honeybee colony: a sequence of actions taken by the workers and the queen. At each step, the bees have an opportunity to influence the colony's investment in drones. Arrows indicate typical sequences of events. Drone cells are larger than worker cells, and drones are normally reared in these larger cells (I). The worker and drone cells are used either for food storage (nectar and pollen; darker cells in diagram) or are cleaned in preparation for rearing brood (II). The queen typically lays unfertilized (drone) eggs into drone cells, and fertilized (female) eggs into worker cells (III). Workers then tend the larvae, but don't necessarily raise all of the larvae to adulthood (IV). Adult drones remain colony members until they mate with a virgin queen in the population, die, or are evicted by workers (V). In this study, we focus primarily on the modulation of drone production that arises from actions taken by the queen (step III).

empty cells that can receive an egg. The queen may then use these empty cells for depositing eggs. She produces a female or male egg depending on whether or not she fertilizes the egg, and generally lays female eggs in worker cells and male eggs in drone cells (Ratnieks and Keller 1998). The queen sometimes lays eggs in a drone to worker ratio that deviates from the ratio of available drone to worker cells (Henderson 1991), suggesting that she might be able to control the sex of her offspring by selecting what type of comb to use. Next, the larvae are tended by the worker bees, who can decrease investment in drones or workers by failing to provision larvae or by consuming them (Free and Williams 1975; Schmickl and Crailsheim 2001; Sasaki et al. 2004). Finally, the drones that reach adulthood may be evicted from the colony by the worker bees in the late fall or when foraging conditions are poor (Morse et al. 1967; Free and Williams 1975). Because workers initially construct the brood cells and can later alter any queen investment patterns by decreasing the number of immature or adult drones, it is widely assumed that workers control colony drone production.

The value of drones varies with environmental factors. In order to increase colony efficiency by minimizing the number of drones that will be destroyed by the workers, a queen might be expected to adjust her egg-laying patterns to achieve her colony's current optimum investment in drones. Season and energy budget are important factors that affect colony drone production (Free and Williams 1975; Seeley and Mikheyev 2003), and there is some evidence that the queen alters her drone egg production in relation to these (Sasaki and Obara 2001). In addition, colony drone production is inhibited by the presence of drone brood (Free and Williams 1975), indicating that colonies regulate drone production through a negative feedback process. Since this drone brood normally

originates from eggs produced by the queen, queens might use oviposition history or the presence of drone brood as an indicator of how many drone eggs to lay. In accordance with this, Sasaki et al. (1996) provide evidence that suggests that honeybee queens decrease subsequent investment in drones if they recently laid drone eggs. However, because the authors used the same queens in multiple treatment conditions staged over time, their data failed to exclude both season and colony energy budget as explanatory variables for the observed queen egg-laying response. Thus, whether queens can adaptively regulate drone egg production remains an open and relevant question.

In this study, we used a between-subjects design to test whether honeybee queens regulate investment in drones. We manipulated the previous egg-laying experience of queens by either allowing or preventing them from laying drone eggs for a period of time, and we then provided them the opportunity to lay both drone and female eggs. If the queen's production of drone eggs is regulated by negative feedback, we expected that queens who were allowed to lay drone eggs for a period of time would subsequently lay fewer drone eggs compared to queens who were initially prevented from laying drone eggs.

## Methods

### Honeybee colonies

We conducted this study in the summers of 2004 and 2005 in the countryside surrounding the Michigan State University campus in East Lansing, Michigan. Twenty four colonies of Italian honeybees, *Apis mellifera ligustica*, were used; 12 each summer. Because each colony had been purchased as a package in the late spring of the year it was used in the study, no colony was used twice, and all were approximately the same size (~13,000 bees). Each colony was housed in a standard Langstroth hive consisting of one deep hive body with ten frames of worker comb. By initially providing the colonies with worker comb only, we ensured that drone production occurred mainly during the experimental periods.

#### General methods

The general strategy was to confine half of the queens to drone comb and half to worker comb for a period of time, and then to compare the egg-laying patterns of the two groups of queens later, when both types of comb were available.

We randomly assigned colonies to one of two treatments, drone comb (DC) or worker comb (WC). The treatment labels refer to the type of comb to which we confined the colony's queen before moving her onto a frame with both types of comb. Therefore, before they were moved, queens in WC colonies (WC queens) could not produce viable drone offspring whereas queens in DC colonies (DC queens) could. These treatments were applied on June 18 in 2004 and on June 16 in 2005. We label those dates as "day 0" of the experiment.

On day 0, we confined each queen to a frame of the randomly assigned type of treatment comb (drone or worker). The cage used to confine the queens was made from queen excluder material, which has small openings through which worker bees can freely

pass but the larger queen cannot. We then placed the cage in the colony and positioned it toward an edge of the broodnest, which is where colonies normally rear both drone brood and worker brood.

On day 4, we verified that both the WC and DC queens had laid many eggs on the treatment frame (average proportion of frame area covered with eggs, WC: 0.58, range 0.21-0.91, DC: 0.48, range 0.12-0.84). We then transferred each queen onto a new frame composed of equal areas of drone and worker comb (the choice frame). We placed the choice frame into the queen excluder cage and placed the cage at the other edge of the broodnest. Queens are able to switch between laying drones and workers multiple times while successively laying eggs (Sasaki et al. 1996), and this occurs over just a few minutes or hours (Wharton, unpublished data). Following the methods of Sasaki et al. (1996), we allowed the queens to lay eggs on this choice frame for 24 hours.

On day 5, after we removed each queen from the cage and placed her back into the rest of the colony, we estimated (see below) the number of drone and worker eggs on the choice frame. We used an estimate rather than an actual count because it was faster and thus reduced the exposure of the eggs to the hot summer weather. After making this estimate, we placed the cage containing the choice frame back in the colony. This prevented the queen from laying additional eggs on this frame, but allowed the workers to provision larvae that hatched from these eggs. We removed the queen excluder cage from the colonies on day 7 and 15 of the study in 2004 and 2005, respectively.

To estimate the numbers of drone and worker eggs laid in the choice frame, we covered the frame with a grid made from  $1.27 \times 1.27$  centimeter hardware cloth, and counted the number of grid squares containing at least one cell with an egg in the drone

comb portion of the choice frame and the number of grid squares containing at least one cell with an egg in the worker comb portion of the choice frame. This gave us an estimate of the area of drone and worker comb covered with eggs; this technique is commonly used to obtain estimates of areas of comb covered with young brood (Jeffree 1958). Since drone and worker cells are different sizes, we multiplied the area of drone and worker comb by the number of cells in that area (using the convention of 2.60 drone cells per cm<sup>2</sup> and 4.29 worker cells per cm<sup>2</sup>; Page and Metcalf, 1984) to obtain an estimate for the number of drone and worker eggs laid by the queen, providing a more intuitive notion of queen behavior. This transformation from area to eggs is likely to give a slight overestimate of the actual number of eggs, but the amount of overestimation should be similar for both treatment groups. Because this transformation is applied equally across treatment groups and is linear, it does not affect our analyses or conclusions.

As we were collecting the data on eggs laid by the queen, we noticed that some of the cells in the choice frame had been filled with nectar, which would have deterred queens from laying eggs in these cells. We used the method just described to estimate the maximum area (in  $cm^2$ ) of drone and worker comb that was used for nectar storage.

After the eggs on the treatment and choice frames reached the pupal stage (and before these pupae emerged as adults), we removed the frames from each hive and took digital photographs of them. We used these photographs to count the number of drone and worker pupae (indicated by capped cells; worker pupae are indicated by flat cappings and drone pupae are indicated by raised cappings) on both the treatment and choice frames for each colony. We noticed that there were some worker pupae on the drone comb treatment frames and on the drone comb half of the choice frames, indicating that

the queen had occasionally laid worker eggs in drone cells (as reported in Page et. al. 1993). This occurrence was extremely rare on the choice frames: of the colonies that had pupae on the drone portion of the choice frame, the proportion of these pupae that were worker pupae was less than 0.029 in all but three of the colonies. In two of the three remaining colonies, the proportions of pupae that were worker were 0.051 and 0.105, and the remaining colony had only one pupa (a worker pupae in drone cells was so rare on the choice frames. Since the occurrence of worker pupae in drone cells was so rare on the choice frames, we are confident that our estimate of the number of drone eggs on the choice frames closely approximates the actual number of drone eggs present. In 2004, we photographed the treatment frames on day 17 and the choice frames on day 20. In 2005, we photographed both the treatment and choice frames on day 15.

# Drone departure rates

Although we did not provide our colonies with any drone comb prior to the start of the experiment, colonies often build small patches of drone comb in gaps and spaces within the hive. Our colonies had done so and consequently had reared a small number of adult drones before the start of the experiment. Since the presence of adult drones might influence a colony's future production of drones (Rinderer et al. 1985) and therefore might influence the egg-laying decisions of the queen, we measured whether the DC and WC colonies had similar numbers of adult drones near the beginning of the study. To estimate the prevalence of adult drones, we measured the rate of drone departures from each colony. An observer sat near the entrance of a hive, recorded the number of drones

leaving during a short period of time (either 1 or 5 minutes), and visited each hive in a fixed order until each was visited for 10 minutes total. We then converted these counts to the rate of drone departure per minute. We made these observations on sunny afternoons, when adult drones in the hive embark on either practice or real mating flights. In 2004, we performed these counts on days 1 and 7 of the study. In 2005, we performed these counts on days 3 and 7 of the study. Thus, for each colony, we obtained two rates of drone departures, which were averaged to obtain the colony's drone departure rate.

#### Statistical analysis

Every queen laid eggs on both the treatment and choice frames, so we included all of the colonies (n = 24) in our analysis. Since we collected data in both 2004 and 2005, we first checked for differences in our data between years. For each treatment group, we found no differences in our dependent variables across years, so we pooled our data from 2004 and 2005 for the statistical analyses.

To test whether DC and WC queens differed in egg-laying decisions on the choice frame, we used a MANOVA to test for the effect of treatment on queen decisions on the choice frame (number of worker eggs, number of drone eggs). A queen may alter her production of drone eggs through two methods: by skewing the sex ratio of her eggs while holding total eggs constant, or by increasing her total production of eggs while holding her egg sex ratio constant. Whereas a test for treatment differences in egg sex ratio can only capture the former method, a MANOVA, which tests for differences in

centroid locations among treatment groups, can capture either method. We used post-hoc analyses to provide further insights into the decisions made by the queens.

To assess whether the egg patterns we observed on the choice frame may have arisen because of worker decisions to fill areas of comb with nectar, we used Student's ttest to assess the difference between DC and WC colonies with respect to the areas of both worker comb and drone comb that were used for nectar storage.

To test whether DC and WC colonies differed in pupae-rearing patterns on the choice frame, we used a MANOVA to test for the effect of treatment on colony decisions on the choice frame (number of worker pupae, number of drone pupae). We used posthoc tests to provide further insights into the colony decisions.

To assess whether worker honeybees might have altered patterns of queen investment differentially across treatments, we used Student's t-test to determine the difference between DC and WC colonies with respect to both drone mortality and worker mortality on the choice frame. We defined drone mortality and worker mortality as the proportion of drone and worker eggs, respectively, that did not reach the pupal stage. We obtained each hive's measure of drone mortality by taking the difference between the numbers of drone eggs and drone pupae and dividing that by the number of drone eggs; worker mortality was obtained in a similar way with worker eggs and pupae. For this analysis, we used data from 2005 only, because in that year we kept the choice frame in the queen excluder cage for long enough to ensure that any pupae we counted on our digital pictures were from eggs that the queen laid while she was confined to that frame.

Finally, we used Student's t-test to determine the statistical significance of the difference between the DC and WC colonies with respect to both drone departure rate

and the total investment in pupal males by the colonies (the sum total of drone pupae on the treatment and choice frames).

All analyses were performed using MATLAB 7.0. Significance was set at the 0.05 level, and results are reported as mean  $\pm 1$  standard deviation.

#### Results

Does previous comb type influence the queen's egg-laying patterns?

Once moved to the choice frames, WC queens and DC queens differed in their egg-laying decisions (MANOVA, Wilks'  $\Lambda_{1,22} = 0.717$ , P = 0.030; Figure 2.2). Specifically, WC queens laid a greater number of drone eggs than did DC queens (number of drone eggs, WC queens: 925.0 ± 665.4, DC queens: 337.9 ± 390.3; F<sub>1,22</sub> = 6.950, P = 0.015). However, WC and DC queens did not differ in the number of worker eggs they produced (number of worker eggs, WC queens: 558.7 ± 534.0, DC queens: 762.3 ± 466.1; F<sub>1,22</sub> = 0.995, p = 0.329).

To see if the difference in queen behavior was due to queens skewing their egg sex ratio, we compared the egg sex ratio (male eggs divided by total eggs) across treatment groups. WC queens tended to lay a more drone-biased sex ratio ( $0.59 \pm 0.40$ ) than did DC queens ( $0.35 \pm 0.36$ ), although this trend was not significant ( $F_{1,22} = 2.340$ , P = 0.140). To see whether the difference in queen behavior was due to queens altering their total production of eggs, we compared the number of eggs produced across treatment groups. WC queens laid a significantly larger number of total eggs than did DC



Figure 2.2. Mean ( $\pm$ SE) number of drone eggs plotted against mean ( $\pm$ SE) number of worker eggs on the choice frame for queens previously on worker comb (WC) and queens previously on drone comb (DC). WC queens laid more drone eggs but the same amount of worker eggs as DC queens. The lines represent the egg sex ratio vector on the choice frame; the slope is the average ratio of drone eggs to worker eggs (dashed line for WC queens, dotted line for DC queens). WC queens and DC queens did not produce significantly different egg sex ratios.

queens (total eggs, WC queens:  $1483.8 \pm 337.4$ , DC queens:  $1100.2 \pm 439.7$ ;  $F_{1,22} = 5.747$ , P = 0.025). This egg production, which occurred over a period of 24 hours, is consistent with reported values of queen egg production, which usually range between 1000 and 2000 eggs per 24 hours (Nolan 1925; Seeley 1985).

#### Do the egg-laying patterns on the choice frame result from the actions of the workers?

The results we reported in the above section could arise from the workers rather than the queen if the egg-laying patterns were simply a byproduct of one of the following (refer to Figure 2.1): worker decisions to selectively clean drone or worker cells, worker decisions to selectively fill drone or worker cells with nectar, or worker decisions to selectively destroy the queen's drone eggs or worker eggs. We address each of those possibilities in this section.

First, the queen's egg-laying pattern might arise from worker decisions to selectively clean drone or worker cells. Workers clean cells by removing debris such as old cocoons, and this cleaning process prepares a cell to receive an egg from the queen (Winston 1987). One way to control for this possibility would be to confine the queen to comb in the absence of workers, but workers tend the queen in many ways, including feeding her. When workers are absent queens lay very few eggs at all (Wharton, unpublished data), but that is a highly unnatural situation and need not have anything to do with the effects of the workers on the cells. In our study, the frames we used had been cleaned by other colonies and stored indoors before they were used for our experiment, so were most likely already prepared to receive eggs from the queens. Even if workers do

play a role in preparing cells for egg-laying, previous work has demonstrated that workers do not differentially prepare worker or drone cells (Sasaki et al. 1996). In summary, no evidence suggested that workers in our study selectively prepared drone or worker cells for the queen.

Second, the queen's egg-laying pattern might arise from worker decisions to selectively fill drone or worker cells with nectar. For example, if the workers in WC colonies filled fewer drone cells with nectar than did the workers in the DC colonies, the significant treatment difference in drone egg production might arise because WC queens had more cells in which to lay drone eggs than did DC queens. However, the area of drone comb filled with nectar did not differ between the two treatment groups (area of drone comb filled with nectar, WC colonies:  $17 \pm 20$  cm<sup>2</sup>, DC colonies:  $25 \pm 34$  cm<sup>2</sup>; t = 0.659, df = 22, P = 0.516; Figure 2.3). Similarly, the area of worker comb filled with nectar did not differ between the two treatment groups (area of worker comb filled with nectar, WC colonies:  $325 \pm 187$  cm<sup>2</sup>, DC colonies:  $219 \pm 126$  cm<sup>2</sup>; t = 1.625, df = 22, P = 0.118; Figure 2.3). Furthermore, since each choice frame had 825.8  $\text{cm}^2$  of worker comb and 825.8 cm<sup>2</sup> of drone comb, queens in both treatment groups still had plenty of available cells in which they could lay eggs. This trend was particularly notable in the drone comb section of the frame, where on average less than three percent of the total area of drone comb was used for nectar storage, leaving almost all of the drone cells available to the queen. Thus, queens were able to make egg-laying decisions independently of worker decisions to fill cells with nectar.

Third, the egg-laying patterns we observed might have resulted from worker decisions to selectively destroy some of the drone eggs. For example, our observation



Figure 2.3. Mean ( $\pm$ SE) area of drone comb (light bars) and worker comb (dark bars) filled with nectar in the choice frames for WC colonies and DC colonies. The choice frames had 825.8 cm<sup>2</sup> drone comb and 825.8 cm<sup>2</sup> worker comb. WC colonies and DC colonies did not differ in the area of drone comb filled with nectar or in the area of worker comb filled with nectar.

that there were more drone eggs in WC colonies than in DC colonies might be explained by a tendency of the workers in DC colonies to selectively destroy drone eggs. We sampled the eggs when they were 0-24 hours old, so we cannot exclude the possibility that workers removed drone eggs before we took our estimates. However, in order for the workers to selectively destroy drone eggs, they would need to perceive the sex of each egg. They might distinguish the sex of eggs based on chemical cues, but there is no difference in removal rates for drone and worker eggs when both are transferred into drone cells (Oldroyd and Ratnieks 2000). Furthermore, there is evidence that workers cannot use brood pheromones to distinguish between drone and worker larvae until seven days after the queen has laid eggs (Sasaki et al. 2004). Alternatively, workers might distinguish the sex of eggs based on cell size alone, but Sasaki et al. (2001) observed food-starved observation hives and did not witness any cannibalization of eggs in drone cells. Additionally, Woyke (1977) demonstrated that there was no difference in removal rates for eggs in drone and worker cells during the spring or summer (when our study was conducted), and although he found selective removal of immature drones in the spring, a sex-specific difference in removal rate didn't occur until the larval stage. For all of these reasons, we find it unlikely that workers in our study were selectively removing drone eggs. In general, whether social insect workers can distinguish the sex of eggs remains an open question in social insect biology (Passera and Aron 1996), and is an attractive area for future research. Other social insect studies have measured egg sex ratios from eggs that were 0-96 hours old and had been exposed to workers (Aron et al. 1994; Aron et al. 1995; Sasaki et al. 1996; Sasaki and Obara 2001), so the methods in our study are consistent with other studies that examine queen egg-laying decisions.

Because it is unlikely that workers in different treatment groups differentially prepared cells, filled cells with nectar, or destroyed eggs, the egg-laying patterns we observed on the choice frames were unlikely due to the actions of the workers, but instead were due to the actions of the queen.

## Does previous comb type influence pupae-rearing patterns of the colony?

On the choice frames, WC colonies and DC colonies differed in their pupaerearing patterns (MANOVA, Wilks'  $\Lambda_{1,22} = 0.639$ , P = 0.009; Figure 2.4). Specifically, WC colonies raised a greater number of drones than did DC colonies (number of drone pupae, WC: 549.6 ± 351.8, DC: 134.0 ± 210.8; F<sub>1,22</sub> = 12.322, P = 0.002) and also produced a more male-biased sex ratio (pupal sex ratio, WC: 0.58 ± 0.36, DC: 0.24 ± 0.26; F<sub>1,22</sub> = 5.701, p = 0.028). WC and DC colonies did not differ in the number of worker pupae they produced (number of worker pupae, WC: 458.0 ± 453.6, DC: 475.6 ± 544.7; F<sub>1,22</sub> = 0.007, p = 0.932) or in the total number of pupae reared on the choice frame (number of total pupae, WC: 1007.6 ± 564.3, DC: 609.6 ± 628.0; F<sub>1,22</sub> = 2.667, p = 0.117).

Thus, just as WC queens had produced more drone eggs on the choice frames than did DC queens, WC colonies produced more drone pupae on the choice frames than did DC colonies. This result indicates that the negative feedback pattern of drone egg production persisted at the colony level.



Figure 2.4. Mean ( $\pm$ SE) number of drone pupae plotted against mean ( $\pm$ SE) number of worker pupae on the choice frame for colonies with queens previously on worker comb (WC) and colonies with queens previously on drone comb (DC). WC colonies raised more drone pupae but the same amount of worker pupae as DC colonies. The lines represent the pupal sex ratio vector on the choice frame; the slope is the average ratio of drone pupae to worker pupae (dashed line for WC colonies, dotted line for DC colonies). WC colonies produced a more male-biased sex ratio than did DC queens.

## Does previous comb type influence drone mortality or worker mortality?

These results are for data taken in 2005. We did observe both drone and worker mortality on the choice frame, meaning that our estimates of drone and worker eggs were higher than the numbers of drone and worker pupae we counted. Specifically, the WC colonies failed to rear  $54.2 \pm 13.9$  percent of the drone eggs and  $53.2 \pm 12.0$  percent of the worker eggs on the choice frames, and the DC colonies failed to rear  $57.0 \pm 28.7$ percent of the drone eggs and  $60.0 \pm 24.1$  percent of the worker eggs on the choice frames. Some of this estimated mortality is most likely a byproduct of our overestimation of the number of eggs (see methods), which is expected to be equal for the two treatment groups. The remainder of the estimated mortality might have arisen from the failure of the worker honeybees to rear some of the drone and worker larvae. If workers were failing to rear brood in a treatment-dependent manner, we should see a difference in mortality between the treatment groups. However, treatment had no effect on the amount of drone mortality (t = 0.190, df = 7, p = 0.855) or the amount of worker mortality (t =0.619, df = 10, p = 0.550). This suggests that any decisions that the workers made about drone or worker removal were independent of the queen's recent egg-laying decisions. Therefore, the net differences between treatments in the pupae-rearing patterns of the colonies can be at least partially attributed to actions of the queens.

Did the colonies differ in their investment in drones throughout the study?

We measured a drone departure rate at the beginning of the experiment of  $0.16 \pm 0.37$  drones per minute in the WC colonies and  $0.27 \pm 0.25$  drones per minute in the DC colonies. These rates are not significantly different (t = 0.816, df = 22, P = 0.423), indicating that the two treatment groups had roughly the same number of adult drones present at the beginning of the experiment. Therefore, any treatment differences in queen egg-laying decisions or colony brood-rearing patterns did not result from a difference in abundance of adult drones in the colonies.

By the end of the experiment (day 20 in 2004 and day 15 in 2005; see methods), we could determine the total number of drone pupae reared throughout the study by summing the drone pupae on both the treatment and the choice frames. WC and DC colonies did not differ in this total (total number of drone pupae produced during the experiment, WC colonies:  $564.8 \pm 343.4$ , DC colonies:  $593.3 \pm 548.5$ ; t = 0.153, df = 18.5, P = 0.880). Therefore, even though WC colonies were given only one half of a frame of drone comb whereas DC colonies were given three times that amount, the treatment groups produced a similar final number of pupal drones. This suggests that the colonies regulated their production of drones.

#### Discussion

It is often assumed that worker honeybees regulate their colony's investment in drones, since they build the wax cells for rearing drones and also tend to the larvae. The results of this study suggest that honeybee queens also contribute to the regulation of their colony's drone production, through modulation of their egg-laying decisions.

Queens that were prevented from laying drone eggs for a brief period of time (WC queens) later produced more drone eggs than queens who had not been prevented from laying drone eggs (DC queens). As the eggs on the choice frame matured into pupae, there is no evidence that the workers selectively killed the larvae in a treatment-dependent manner, so the heightened production of drone eggs by WC queens (when given a choice of egg type to lay) allowed the WC colonies to "catch up" on the number of drones that were being reared. As a result, both treatment groups reared approximately the same total number of drone pupae throughout the experiment.

Our finding that the colonies invested equally in pupal males over the duration of the experiment is consistent with studies indicating that colonies across a population produce similar amounts of drone brood (Page and Metcalf 1984; Henderson 1991). Our colonies were approximately equal in size and therefore are expected to invest equally in drones. It is remarkable that our colonies were able to accomplish this even though they differed greatly in the amount of drone comb available (DC colonies were provided with three times the amount of drone comb as WC colonies). Therefore, our study provides further evidence that honeybee colonies are adept at regulating drone production.

As for how this regulation of drone production occurs, our study suggests a greater role for the queen than is often assumed. Previous studies have shown that the presence of drone brood suppresses the further production of drone brood in honeybee colonies (Free and Williams 1975); brood production might also be suppressed by a large quantity of adult drones in the colony (Rinderer et. al., 1985; but see Henderson, 1991). Together, these studies suggest that colonies regulate drone production via a negative feedback mechanism. However, these previous studies assessed colony drone production

by counting numbers of drone larvae or pupae, which may reflect a combination of queen and worker decisions. Therefore, these studies do not directly address the role that the queen plays in regulating colony drone production. Our study fills this gap by focusing on whether the queen regulates her drone egg production. We found that the queen compensates for her own production of drone eggs based on the amount of drone eggs she recently laid. Furthermore, the workers in our study did not block (via using cells for nectar storage) or alter (via failing to rear larvae) the queen's decisions in any sort of treatment-dependent manner. Taken together, these results suggest that the regulation of drone brood production at the colony level may emerge at least in part by a negative feedback process of drone egg production by the queen.

Queens might increase their drone egg production by laying a greater total number of eggs, by creating a more drone-biased egg sex ratio, or some combination of these two. In our study, queens did differ in the total number of eggs they laid, which was due to a difference in the number of drone but not worker eggs that were laid. The difference in egg sex ratio was not statistically significant, but we did find a trend in the predicted direction, in that queens who were prevented from laying drone eggs subsequently produced a more drone-biased egg sex ratio. Because we had a relatively low sample size and queen behavior was quite variable, we suspect that this sex ratio trend may reflect a biologically real behavior. In short, honeybee queens can modulate their investment in drones by altering the total number of drone eggs they lay and possibly also by changing their egg sex ratio.

Whether social insect queens can adaptively regulate the egg sex ratio is an important line of future research. This ability has been found in queens of species that

have a high potential for queen-worker conflict over sex allocation (Aron et al. 1995; Keller et al. 1996b; de Menten et al. 2005a; de Menten et al. 2005b). In species that experience this queen-worker conflict, the queen may manipulate her egg sex ratio in order to exercise power, where power is defined as the ability to control reproduction when conflict exists (Beekman et al. 2003; Beekman and Ratnieks 2003). Alternatively, she might manipulate her egg sex ratio in order to influence the colony's allocation to reproduction in agreement with the workers. Future studies that examine whether queens can influence colony male production should attempt to distinguish between these two functional hypotheses.

Our study opens up some fascinating questions that deserve further work. For example, what proximate cues does the queen use to alter her egg-laying decisions in relation to previous egg-laying decisions? As one possibility, a queen might use some type of memory to keep track of previous ovipositions, and could stop producing more drone eggs after she remembers laying many drone eggs. Alternatively, queens might use brood pheromone cues to indicate the amount of drones in her colony, and could stop producing more drone eggs when her chemical sensory system detects a large amount of drone brood. In our study, we did not remove any eggs before allowing queens to make egg-laying choices, so our experimental design does not allow us to distinguish between the hypotheses that queens are using memory or brood pheromones. Identifying the proximate mechanisms by which social insect queens keep track of the colony's abundance of males is an attractive study for future research, because it has implications for the ability of queens to react to male removal by the workers. In species that experience conflict, for example, if workers selectively remove males, we predict that the

queen might not rely on oviposition memory because it will mislead her estimate of the number of developing male larvae.

This work sets the stage for examining how the actions and interactions of the queen and workers influence a colony's investment in reproduction. Investing in males can be likened to a supply chain management issue for the colony, where a major goal should be to produce an adaptive amount of a product (males) through the most efficient use of resources. Brood production should be an efficient process if both the workers and the queen are able to actively and separately respond to environmental conditions that convey information about the value of rearing males. In honeybees, some of these conditions include season, colony size, availability of food, whether or not a queen is present, and the number of drones present (Free and Williams 1975; Page and Metcalf 1984; Seeley and Mikheyev 2003). How do colony members perceive information about these conditions? Which conditions are assessed by the queen, and which are assessed by the workers? Additionally, environmental conditions that favor drone production might change after the queen has laid drone eggs. Will workers modify the previous egg-laying decisions of their queen if environmental conditions suddenly become unfavorable for rearing drones? These questions, which resemble questions from supply chain management, must await further work.

In conclusion, our study identifies a role for the honeybee queen in the regulation of colony drone production. This demonstrates that a social insect queen can influence colony male production even when conflict with the workers is absent. In order to better understand the functional significance of queen and worker decisions, future work should attempt to distinguish between actions that arise from conflict over sex allocation and

those that arise from cooperation and shared control over the colony's investment in reproduction.

# CHAPTER 3 MALE ELIMINATION IN THE HONEYBEE

Wharton KE, Dyer FC, Getty T (In press) Male elimination in the honeybee. Behavioral Ecology. Oxford University Press.

## Introduction

Cooperation and conflict between subunits of a group are pervasive forces at multiple levels of biological organization, from genomes to organisms to animal societies. Understanding how cooperation arises and is maintained in spite of the potential for intragroup conflicts is a central quest in evolutionary biology (Queller and Strassmann 1998; Reeve and Hölldobler 2007). In animal societies, individuals often have competing interests that can lead to potential conflicts over the division of resources or reproduction among society members (Ratnieks et al. 2006). However, it can be tricky to determine the extent to which group member interactions are shaped by conflict because an observed behavior might be influenced by multiple environmental, genetic, and evolutionary factors (Chapuisat and Keller 1999). In this paper we examine a behavior that is often assumed to spring from genetic conflict between the queen and female workers in a social insect colony, the elimination of immature males by the workers. Most studies of male elimination have focused on species in which queenworker conflict is expected to be high. To determine whether genetic conflict is a necessary precondition for male elimination, we studied honeybees (Apis mellifera), a species where such conflict is expected to be minimal, and in which any evidence of male elimination is likely to reflect cooperative resource allocation to male reproductive function.

In the social Hymenoptera (ants, bees, wasps), the queen and workers are potentially in conflict over their colony's sex allocation, or investment in males versus females. Although there are several types of queen-worker conflict (Ratnieks et al. 2006), we consider only conflict over sex allocation in this paper. The potential for this conflict

arises from haplodiploid sex determination (females are diploid; males are haploid), which causes workers to be, on average, more closely related to their sisters than to brothers, whereas the queen is equally related to her daughters and sons (Hamilton 1964). These patterns of relatedness lead to the prediction that workers should generally prefer a less male-biased sex allocation than the queen (Trivers and Hare 1976).

Workers might bias sex allocation in their favor by eliminating a portion of the colony's immature males. Male elimination has been inferred to occur in several ant species, based on observations that the secondary sex ratio was less male-biased than the egg sex ratio (Aron et al. 1994; Aron et al. 1995; Keller et al. 1996a; Passera and Aron 1996; Sundström et al. 1996; Chapuisat et al. 1997; Helms et al. 2000; Rosset and Chapuisat 2006). Since male elimination allows workers to bias sex allocation in their favor, these examples are, perhaps not surprisingly, consistently cited as evidence of a manifest queen-worker conflict (Mehdiabadi et al. 2003; Ratnieks et al. 2006).

Although male elimination is consistent with the predictions of queen-worker genetic conflict, it might also serve a cooperative purpose. Workers have an opportunity to modify their colony's investment in reproduction when they rear brood; these broodrearing decisions might allow the colony to adaptively tailor reproductive effort to resource availability or other environmental conditions (Wharton et al. 2007). For example, workers might eliminate some immature males when colony resources are limited or when the colony is already well-supplied with males. By eliminating males before they extract full developmental costs and begin extracting high adult maintenance costs, workers could enhance overall colony efficiency and success (Chapuisat et al. 1997).

So far, all reported cases of male elimination have been in species where the queen is singly mated and so the potential for conflict is high. Is genetic queen-worker conflict therefore a necessary precondition for male elimination? To determine this, it is important to investigate whether there is selective male elimination in species in which the queen mates multiple times. Multiple mating by the queen reduces the average relatedness among workers and lowers the potential for conflict (Moritz 1985; Pamilo 1991b; Pamilo 1991a). If male elimination were solely a consequence of queen-worker conflict, then we should not see it in such species. Only one study examined a case in which conflict was expected to be low (in wood ant colonies headed by a multiply mated queen); it found no evidence for selective male elimination (Sundström et al. 1996). Clearly, however, there is a need for data from other species.

Honeybees are a good candidate species for testing whether male elimination might spring from cooperation. Honeybee queens are highly polyandrous (Tarpy and Nielsen 2002), so queen-worker conflict is expected to be very low or absent (Moritz 1985; Ratnieks et al. 2006). Additionally, colonies do not rear as many drones (males) as possible but rather regulate their investment in drones (Free and Williams 1975), presumably because drones are costly to produce (Seeley 2002; Seeley and Mikheyev 2003; Hrassnigg and Crailsheim 2005). Conveniently, good estimates of brood survival are possible in honeybees because researchers can reliably and nondestructively determine the sex of eggs: drone eggs are found in large "drone" cells whereas worker eggs are found in small "worker" cells (Ratnieks and Keller 1998). This difference in cell size also lends itself to experimental manipulations; researchers can limit or manipulate a colony's production of drones by controlling the abundance of drone cells in the colony.

In this study, we compared the survival of young immature drone and worker honeybees under two different experimental conditions: one in which colonies had an excess of older drone brood (D+ colonies) and one in which colonies had virtually no additional drone brood (D- colonies). See Figure 1 for the experimental design. This design was chosen because the presence of drone brood (immature drones) inhibits a colony's future production of drones (Free and Williams 1975). The treatments were induced after the queen had laid both drone and worker eggs in order to isolate the influence of worker actions on brood survival. We predicted that if workers adaptively influence the colony's investment in drones by eliminating immature drones, the survival of drone but not worker eggs would be lower in colonies that already were rearing large numbers of drones.

# Methods

## Honeybee colonies

We conducted this study on the Michigan State University campus in East Lansing, Michigan in May and June, 2007. Twenty colonies of Italian honeybees, *Apis mellifera ligustica*, were used. Each colony contained the mother queen and roughly 15,000 workers, and was housed in a standard Langstroth hive consisting of one deep hive body with ten frames of worker comb. By initially providing the colonies only with worker comb, we ensured that drone production occurred mainly during the experiment.

# General methods

Our general strategy was as follows. First, in each of the study colonies we allowed the queen to lay drone and worker eggs in the presence of older drone brood (immature drones). Then, we divided the colonies into two groups, one from which drone brood was removed and the other to which drone brood was added. Finally, we compared the survival of the new worker and drone brood during the period in which the colony's workers rear the larvae. The experimental setup is presented in Figure 1.

To set up the experiment, we placed one frame of drone comb and one frame of worker comb (the "treatment" frames) into each colony. These frames were used to manipulate the abundance of drone brood for the experimental treatments. After ten days, these frames were largely filled with brood consisting of older larvae and some pupae.

To obtain eggs so that we could monitor the brood-rearing decisions of the workers, we inserted into each colony a "focal frame" consisting of drone and worker comb in blocks of equal area. On day 0 of the experiment, we caged the queen on this comb and allowed her to lay eggs for 24 hours. The cage was made of queen excluder material, which consists of small openings that prevent the queen but not the workers from passing through. At the end of this 24-hour egg-laying period, we placed the queen elsewhere in the colony and took a series of photographs that facilitated counting of



Figure 3.1. Experimental design, from a bird's eye view of the moveable frames of comb in a colony. Dark gray bar = focal frame (composed of equal areas of drone and worker comb). Light gray bars = treatment frames (D = drone, W = worker). White bars = other frames in colony (contained mostly worker brood, pollen, honey, and empty worker cells). The dashed line indicates the queen excluder cage and the triangle ( $\Box$ ) indicates the position of the queen. At the beginning of experiment (left), the queen was placed on the focal frame in the queen excluder cage and permitted to lay drone and worker eggs. Next, the queen was placed elsewhere in the colony and each colony was randomly assigned to one of two treatment groups (right). The treatment was applied by exchanging frames between the two groups of colonies. In the drone brood removed (D-) group, the colony lost its drone treatment frame, and a worker treatment frame was inserted in its place. In the drone brood added (D+) group, the colony received an additional drone treatment frame in the place of a worker treatment frame.
numbers of worker and drone eggs laid by the queen. We then returned each of the focal frames to the cage in its colony, allowing worker access but this time with the queen excluded so that no further eggs could be laid on this comb.

To test whether the workers would adaptively modify their colony's investment in drones, we randomly assigned the colonies to one of two treatments, drone brood added (D+) or drone brood removed (D-). Each D+ colony received a drone treatment frame from a D- colony and gave a worker treatment frame to that D- colony in return. Thus, after this treatment was applied, the D+ colonies had two frames of older drone brood whereas the D- colonies had none. We applied this treatment over the course of days 2 and 3.

To quantify larvae for estimating brood survival, we took photographs of the focal frame when the brood had reached the late larval stage and were just prior to capping (day 8; 8-9 days after oviposition). In order to quantify the pupae on the treatment frames, we took photographs of the treatment frames twice: once on day 3 (shortly after the treatment had been applied), and once at the conclusion of the experiment, on day 8 (for the worker treatment frames) and day 9 (for the drone treatment frames).

Before the start of the experiment, our colonies had built small patches of drone comb in gaps within the hive. Consequently, our colonies contained a small background number of adult drones during the experiment. Since adult drones might influence drone production (Rinderer et al. 1985), we recorded the number of drones embarking on mating flights to determine whether the D+ and D- colonies had similar numbers of adult drones (see Seeley 2002). On day 8, an observer sat near the entrance of a hive, recorded the number of drones that left the colony during a 5 minute interval, and visited each hive

in a fixed order until each was visited for 10 minutes total. We used these counts to calculate a rate of drone departures per minute.

### Survival of immature drones and workers

To calculate survival of brood on the focal frame, we used photographs to quantify (blindly) both the eggs initially present and the larvae that remained after our experimental manipulation. We used photographs because direct counts would have taken too long and increased the likelihood that the eggs would die from exposure to the summer heat. The larvae we counted were at a late (5-6 day) larval stage; nearly all larvae that old survive to eclosion (Woyke 1977; Schmickl and Crailsheim 2001; Schmickl and Crailsheim 2002).

Counting eggs from photographs is prone to error because of the small size of the eggs, their lack of color contrast with the wax cells, their position at the bottom of a cell, and various irregularities of the cells themselves. We expected to undercount the actual number of eggs on the focal frame. However, because the tendency to undercount depends mostly on wax comb properties it should not be systematically biased across treatment groups.

Drone or worker survival on the focal frame was estimated from the proportion of eggs in each type of cell that reached the late larval stage. These counts did reveal that we may have undercounted eggs at the beginning. We counted more worker larvae than worker eggs in 3 of our colonies (467 larvae, 443 eggs; 593 larvae, 588 eggs; 235 larvae, 74 eggs), and we counted more drone larvae than drone eggs in 2 of our colonies (728

larvae, 634 eggs; 584 larvae, 573 eggs). For these cases we assigned a worker or drone survival value of 1.

### Statistical analysis

There were no drone eggs on the focal frame in 2 of our 20 colonies, so we excluded those colonies from the analyses. Within each treatment group, all dependent variables were checked for normality using a Lillefors test. For any dependent variable that did not meet the criterion for normality, the appropriate nonparametric test was used for hypothesis-testing. All analyses were performed using MATLAB 7.0, and results are reported as mean  $\pm 1$  standard deviation.

### Results

## Comparisons of the number of eggs on the focal frame and adult drones in the colony

Since the experimental treatment was randomly assigned after the queen had laid eggs on the focal frame, we did not expect any differences between the treatment groups in the number of eggs laid. Consistent with this, D- and D+ colonies did not differ in the number of drone eggs (D- colonies:  $456.2 \pm 318.8$ , D+ colonies:  $333.7 \pm 277.5$ ; t = 0.868, df = 16, P = 0.398) or in the number of worker eggs (D- colonies:  $797.8 \pm 473.9$ , D+ colonies:  $619.1 \pm 275.8$ ; t = 0.978, df = 16, P = 0.343) on the focal frame. Furthermore,

there was no difference in the egg sex ratio (proportion of drone eggs, D- colonies:  $0.38 \pm 0.24$ , D+ colonies:  $0.30 \pm 0.21$ , Mann-Whitney U test, P = 0.489).

We also found that the abundance of adult drones (produced prior to the beginning of the experiment) did not differ between treatment groups (drone departures per minute, D- colonies:  $0.51 \pm 0.60$ , D+ colonies:  $0.60 \pm 0.83$ ; Mann-Whitney U test, P = 0.774).

Because colonies did not differ significantly in the production of drone or worker eggs on the focal frame, or in the abundance of adult drones, any treatment differences in colony brood-rearing patterns cannot be attributed to those factors.

# Comparisons of brood survival

D- colonies and D+ colonies differed with respect to brood survival (consisting of 2 variables: drone survival, worker survival) on the focal frame (MANOVA, Wilks'  $\Lambda_{1,16} = 0.566$ , P = 0.014) (Fig. 2). Specifically, a greater proportion of drone eggs were reared to the late larval stage in D- colonies than in D+ colonies (drone survival, D- colonies:  $0.83 \pm 0.18$ , D+ colonies:  $0.43 \pm 0.34$ ;  $F_{1,16} = 9.29$ , P = 0.008). D- and D+ colonies did not differ in the proportion of worker eggs that were reared to the late larval stage (worker survival, D- colonies:  $0.78 \pm 0.16$ , D+ colonies:  $0.72 \pm 0.25$ ;  $F_{1,16} = 0.22$ , P = 0.642). This indicates that our treatment affected the survival of immature drones but not workers.

To further investigate how our colonies reared worker brood versus drone brood on the choice frame, we examined the relationship between drone survival and worker



Figure 3.2. The survival of worker eggs and drone eggs to the larval stage on the focal frame in colonies that had drone brood removed (open circles) and drone brood added (filled circles). The overall mean ( $\pm$  standard error) survival for each treatment group is indicated by the correspondingly shaded squares and error bars. The survival patterns differed significantly between the two treatment groups (Wilks'  $\Lambda_{1,16} = 0.566$ , P = 0.014). Specifically, the survival of worker eggs to the larval stage did not differ between treatment groups ( $F_{1,16} = 0.22$ , P = 0.642), but the survival of drone eggs to the larval stage was significantly lower in the colonies to which drone brood was added than in colonies from which drone brood was removed ( $F_{1,16} = 9.29$ , P = 0.008).

survival within each treatment group. There was no correlation between drone survival and worker survival in the D- colonies (r = 0.168, p = 0.666). However, in the D+ colonies, drone survival and worker survival tended to be negatively correlated, although this tendency was not statistically significant (r = -0.556, p = 0.120).

Our D+ colonies varied in the number of drone pupae on the treatment frames. We tested whether this variation across the D+ colonies (9 colonies) explained any variation in drone survival on the focal frames in those colonies. We found no correlation between drone survival and the number of drone pupae on treatment frames at the end of the experiment (D+ colonies: r = 0.496, p = 0.175).

To test whether the treatment caused a shift in sex ratio from the egg stage to the larval stage, we calculated the change in sex ratio for each focal frame by subtracting the larval sex ratio from the egg sex ratio. This shift in sex ratio was not significantly affected by the treatment (change in sex ratio; a positive change indicates a less male-biased larval sex ratio, D- colonies:  $-0.03 \pm 0.05$ , D+ colonies:  $0.03 \pm 0.15$ , Mann-Whitney U test, P = 0.063).

## Discussion

This study provides evidence for conditional male elimination by honeybee workers and is one of only a few studies to date that uses an experimental approach to investigate the expression of this behavior. In colonies with an excess of older drone brood, the survival of newly produced drone brood was lower than in colonies with an absence of older drone brood. There was no effect of treatment on the survival of worker

brood (Fig. 2). These patterns of brood survival indicate that honeybee colonies, like individual organisms, regulate investment in male reproductive function.

As for how this regulation occurs, this study points to an important role for the workers. Previous work has shown that the presence of drone brood suppresses the further production of drone brood in honeybee colonies (Free and Williams 1975), suggesting that colonies regulate drone production via a negative feedback mechanism. Colonies manage to do this through some combination of queen and worker decisions. In this study, we isolated and examined worker influences on brood patterns by removing any direct influence of the queen: treatment groups were assigned after the queen laid eggs, and the queen was prevented from laying additional eggs on the focal frame following treatment assignments. Furthermore, the treatment difference in drone but not worker survival indicates that brood rearing decisions by the workers rather than an overall differences in drone survival demonstrate that honeybee workers can modify their colony's investment in immature drones after the queen lays eggs.

From the perspective of the colony, brood production should be an efficient process if both the workers and the queen are able to respond actively and separately to environmental conditions that convey information about the value of rearing males. The results of this study, together with a previous study by Wharton et al. (2007), suggest that both the honeybee queen and workers can influence their colony's investment in males. There is evidence that this shared or joint control over brood production is found in other species, including the harvester ant *Messor pergandei* (Ode and Rissing 2002) and the fire ant *Solenopsis invicta* (Aron et al. 1995; Passera et al. 2001). Shared control might in

some cases reflect an evolutionary tug-of-war between the queen and workers for control over colony sex allocation (Trivers and Hare 1976; Reuter and Keller 2001). This shared control might arise because neither the queen nor the workers are able to gain complete control over sex allocation in species in which queen-worker conflict is expected. For example, in the fire ant, it appears that the queen is constrained by the ability of the workers to eliminate males, and that the workers are in turn constrained by the primary sex ratio produced by the queen (Aron et al. 1995; Passera et al. 2001). In other cases, including in species without queen-worker conflict, shared control might allow colonies to respond flexibly and efficiently to changing environmental conditions such as fluctuations in food availability (Ode and Rissing 2002; Wharton et al. 2007). This cooperative view might help to explain various phenomena such as why the *Linepithema humile* queen continues to produce male eggs outside the period of sexual production even though workers eliminate the vast majority of those eggs (Aron et al. 1994; Passera and Aron 1996).

Sibling cannibalism occurs in other animals, including amphibians and predatory birds, during periods of low food availability. Although this sibling cannibalism is traditionally considered a form of parent-offspring conflict, researchers suspect that it might serve an adaptive purpose for both parents and offspring in that it promotes a better distribution of limited resources among offspring (Mock and Forbes 1995). Similarly, parent-offspring conflict between a social insect queen and workers has typically served as an explanation for the ability of social insect workers to modify their colony's investment in males (Mehdiabadi et al. 2003; Ratnieks et al. 2006). Indeed, the results from Sundstrom et al. (1996) lend strong support to a role for such conflict in some

colonies of the wood ant *Formica exsecta*. Until our study, all known instances of male elimination in social insects have been reported in species with a high potential for queen-worker conflict (Aron et al. 1994; Aron et al. 1995; Keller et al. 1996a; Passera and Aron 1996; Sundström et al. 1996; Chapuisat et al. 1997; Helms et al. 2000; Rosset and Chapuisat 2006). Our evidence for male elimination in honeybees, a species with little or no potential for queen-worker conflict (Moritz 1985; Ratnieks et al. 2006), suggest that it might be more appropriate to evaluate its function in the context of overall colony performance.

Colony performance is shaped in large part by the ways in which resources are allocated between individuals within the society. The ways in which resources are distributed to brood are sometimes likely to provide benefits in terms of colony efficiency and performance rather than in terms of direct benefits to kin (Chapuisat et al. 1997; Aron et al. 2001; Linksvayer 2008). Our results are consistent with the notion that male elimination in honeybees allows workers to adaptively modify the queen's egg laying decisions in a way that increases colony efficiency. By eliminating excess male brood, the workers might channel resources to more pertinent aspects of colony reproduction or survival, such as additional females or an increased ability to retain colony food reserves. Although we did not measure such life history measures of colony performance in our study, previous studies with honeybees suggest that rearing drones does not trade off with a colony's production of workers, but might negatively impact the colony's ability to stockpile honey for winter survival (Allen 1963; Seeley 2002).

A major question emerging from our study is whether an ability of the social insect queen or workers to influence colony sex allocation reflects within-colony genetic

conflict or shared interests over the colony's resource allocation to male reproductive function. Because a mixture of the two explanations might apply in a species with a high potential for queen-worker conflict, disentangling the relative effects of each might be challenging. To do so, researchers must consider both environmental and genetic factors that favor conflict or cooperation within groups (Ratnieks and Reeve 1992; Reeve and Hölldobler 2007; Linksvayer 2008). These environmental and genetic influences on colony dynamics should be teased apart and investigated using manipulative experiments (Sundström et al. 1996; Chapuisat and Keller 1999). Finally, researchers should consider proximate factors, such as the costs of brood manipulation, that might constrain the ability of the queen and workers to influence sex allocation (Nonacs and Carlin 1990; Chapuisat et al. 1997; Chapuisat and Keller 1999; Beekman et al. 2003; Beekman and Ratnieks 2003; Reuter et al. 2004).

In conclusion, social insect biologists should exercise caution when interpreting male elimination as being indicative of a manifest queen-worker conflict. We echo the plea of Chapuisat and Keller (1999) for additional manipulative experiments, which should lead to a better understanding of the evolutionary factors that promote conflict and cooperation and the ways in which those forces shape social dynamics.

# CHAPTER 4 THERE IS MORE TO SEX ALLOCATION THAN THE SEX RATIO

#### Introduction

Darwin (1874) was puzzled by how natural selection could shape the ability of an organism to adjust its offspring sex ratio, or the proportion of its offspring that are male. Over 130 years later, work on sex ratios has greatly advanced our understanding of how organisms respond to variable reproductive opportunities, including competition with relatives for mates (Hamilton 1967) or resources (Clark 1978), environmental conditions (Trivers and Willard 1973), and within-family conflicts (Trivers and Hare 1976). Sex ratio theory is successful due largely to the development of models that generate testable qualitative and quantitative predictions for behavior (West et al. 2000). However, sex ratio models collapse two dimensions of information [number of males, number of females] to one dimension [number of males/(number of males + number of females)], discarding information about a potentially important aspect of reproductive investment: clutch size. In this paper we assert that clutch size is often an important dimension of reproductive effort and that biologists can gain a better understanding of their study system by focusing on two-dimensional sex allocation decisions rather than on onedimensional sex ratio decisions.

Clutch size, or the sum total of male and female offspring, is an important fitness component of animals (Lack 1947). Life history theory predicts that an iteroparous organism might increase its fitness by producing more offspring during a better than

average reproductive opportunity (Stearns 1992). This theory has been supported by empirical work in a wide range of taxa including birds, insects, and mammals (West and Sheldon 2002; Holand et al. 2006). Recent empirical evidence suggests also that females in some species have the ability to adjust both their offspring clutch size and sex ratio (Olsson et al. 2005; Dubois et al. 2006). Despite the potential importance of clutch size, it is often ignored or treated only as a nuisance covariate in sex ratio research (Krackow and Tkadlec 2001; Hardy 2002). In fact, many researchers statistically control for differences in clutch sizes rather than treat clutch size as potentially important. Increasingly, however, researchers are recognizing that clutch size is not irrelevant, and that in many cases sex ratio and clutch size are interdependent factors that should be examined simultaneously (Nagelkerke 1994; Greeff 1997).

The theories of optimal offspring sex ratio and clutch size are unified in sex allocation theory. Sex allocation is defined as the allocation of resources to male versus female reproductive function (Charnov 1982). Although sex allocation covers many topics in biology, in this paper we focus on the area of sex allocation research that refers to an organism's total investment in male versus female offspring. An organism's sex allocation can be characterized by two simple variables: the number of male [m] and number of female [f] offspring that it produces. Note that these two variables fully determine both sex ratio [m/(m+f)] and clutch size [m+f]. In contrast to sex ratio theory, sex allocation theory therefore asserts that a clutch of 3 males and 6 females is not equivalent to a clutch with 4 males and 8 females. Despite the potential usefulness of examining the raw numbers of male and female offspring, publication titles reveal that biologists tend to think primarily in terms of sex ratios rather than sex allocation (Figure

4.1). We suggest that our understanding of sex allocation will be aided by applying models and analyses that use the raw *numbers* of males and females, rather than the *ratio* of males to females, as the primary variables of interest.

Our goal in this paper is to convince readers that analyses of reproductive decisions should consider both allocation to sex ratio and clutch size (or equivalently the total number of males and females) before reducing reproductive decisions to the single dimension of sex ratio. To illustrate this point, we use existing datasets documenting offspring production by two species of haplodiploid insects: honeybee queens (Wharton et al. 2007) and parasitoid wasps (Shuker et al. 2004). The results from both examples demonstrate that analyzing sex allocation behavior leads to fundamentally different biological conclusions than those drawn from analyzing sex ratio behavior.

#### Example 1: Offspring sex allocation by honeybee queens (Apis mellifera)

#### Overview of study

Social insect colonies, comprised of many individuals, must allocate resources to life history traits like growth, survival, and reproduction. How colonies achieve this through the actions and interactions of colony members remains a puzzle (Bourke and Franks 1995). In a recent study, Wharton et al. (2007) showed that the honeybee queen is able to influence her colony's sex allocation by influencing her colony's regulation of male (drone) production. Here we briefly review the details of that study and highlight the differences that arise from analyzing the queen's ability to influence her colony's sex allocation versus sex ratio.



Figure 4.1. Number of published articles (between the years 1965 and March 2008) listed in the ISI Web of Science that include either the term "sex ratio(s)" or "sex allocation" in the article title. The data in this figure are limited to three journals that specialize in behavioral ecology: Behavioral Ecology and Sociobiology, Animal Behavior, and Behavioral Ecology.

Wharton et al. (2007) tested whether the honeybee queen is able to use previous egg-laying experience to regulate her production of male and female eggs. To do this, the researchers either allowed queens to lay male eggs, or prevented them from doing so, for a period of time, and examined their subsequent tendency to lay male or female eggs. Refer to Wharton et al. (2007) for specific methodological details.

#### Results

To test whether the queens in the two treatment groups differed in their egg sex ratio (number of male eggs divided by the total number of eggs), we used an ANOVA. Queens that had previously been prevented from laying drone eggs (WC queens) produced an egg sex ratio (mean  $\pm$  1 standard deviation) of 0.59  $\pm$  0.40, whereas queens that had previously laid drone eggs (DC queens) produced an egg sex ratio of 0.35  $\pm$  0.36. These egg sex ratios were not significantly different (F<sub>1,22</sub> = 2.340, P = 0.140; Figure 4.2).

To test whether the queens in our two treatment groups differed in their egg sex allocation (number of female eggs, number of male eggs), we used a multivariate analysis of variance (MANOVA). A MANOVA tests for differences in the centroid locations of the dependent variables among the treatment groups. We then used post hoc analyses to provide further insights into the decisions of the queens.

The egg sex allocation produced by the queens was significantly affected by the experimental treatment (Wilks'  $\Lambda_{1,22} = 0.717$ , P = 0.030; Figure 4.3). Specifically, WC queens laid a greater number of drone eggs than did DC queens (number of drone eggs,



Figure 4.2. Mean ( $\pm$  SE) sex ratio of honeybee queens that had previously been prevented from laying drone eggs (WC queens) and queens that had previously been allowed to lay drone eggs (DC queens). The egg sex ratios are not significantly different (F<sub>1,22</sub> = 2.340, P = 0.140).



Figure 4.3. Mean ( $\pm$  SE) sex allocation of honeybee queens that had previously been prevented from laying drone eggs (WC queens) and queens that had previously been allowed to lay drone eggs (DC queens). The egg sex allocation patterns are significantly affected by the treatment (Wilks'  $\Lambda_{1,22} = 0.717$ , P = 0.030). WC queens laid more drones eggs but the same amount of worker eggs as DC queens (see text).

WC queens:  $925.0 \pm 665.4$ , DC queens:  $337.9 \pm 390.3$ ;  $F_{1,22} = 6.950$ , P = 0.015).

However, WC and DC queens did not differ in the number of worker eggs they produced (number of worker eggs, WC queens:  $558.7 \pm 534.0$ , DC queens:  $762.3 \pm 466.1$ ;  $F_{1,22} = 0.995$ , p = 0.329). Overall, WC queens laid a significantly larger number of total eggs than did DC queens (total eggs, WC queens:  $1483.8 \pm 337.4$ , DC queens:  $1100.2 \pm 439.7$ ;  $F_{1,22} = 5.747$ , P = 0.025).

#### Discussion

The conclusions that we draw from this study are fundamentally different depending on whether we analyze the reproductive decisions with a sex allocation or sex ratio approach. The offspring sex allocation of honeybee queens was influenced by previous egg-laying experience. Specifically, queens who initially laid drone eggs subsequently laid fewer drone eggs than the queens who were initially prevented from producing drone eggs. This striking pattern was revealed by our test for an effect of treatment on egg sex allocation (number of worker eggs, number of drone eggs). However, when we discarded information and collapsed our data to the single dimension of sex ratio (drone eggs/total eggs), our test failed to find any significant differences between the treatment groups.

There are biological reasons to suspect a discernable treatment difference in sex allocation but not sex ratio behavior in honeybees. Colonies that are not allowed to invest in drones do not display an increased investment in workers (Allen 1963; Page and Metcalf 1984), suggesting that there is not a colony-level tradeoff between producing drones and workers. Instead, there appears to be a tradeoff between a colony's investment in drones and the size of its honey reserve, a key determinant of overwinter

survival (Seeley 2002). Since a colony's investment in drones likely does not directly trade off with its investment in workers, examining a colony's sex ratio might not be biologically appropriate. Instead, we suggest that researchers should examine a colony's joint investment in drones and workers (sex allocation), which provides a more complete picture of the colony's joint investment in reproduction and growth.

# Example 2: Offspring sex allocation by female parasitoid wasps (Nasonia vitripennis)

#### Overview of study

Local mate competition (LMC) theory predicts that when populations are structured so that only female offspring disperse, a mother should produce a femalebiased clutch of offspring (Hamilton 1967). This female-biased clutch serves to reduce the local competition between brothers for mates. When two or more females share the same patch, this theory predicts that the optimal offspring sex ratio will depend on the extent to which the females are related. Specifically, if females are able to assess their relatedness, females that are more closely related to each other are predicted to lay a more female-biased clutch of offspring than are unrelated females (Frank 1985).

Shuker et. al (2004) assessed the effect of patch partner relatedness on sex ratio behavior in the gregarious parasitoid wasp *Nasonia vitripennis*. The researchers assigned mated females to one of three experimental treatments: A) sisters that were from the same host and therefore had environmental cues about relatedness, B) sisters that were from different hosts and therefore had no environmental cues about relatedness, and C) unrelated females. The researchers replicated these experiments using wasps from two strains: HV236 and HV307. They recorded the number and sex of all offspring that emerged from the hosts (and therefore both wasps combined), and converted these numbers into sex ratios (number of male offspring/total number of offspring) for statistical analyses. Refer to Shuker et. al (2004, Experiment 1), for the detailed experimental methods and statistical analyses.

Here we compare the sex ratio analyses of Shuker and his collagues with our reanalysis of the data in that study. Our analysis focuses on the sex allocation behavior of the wasps, i.e. the numbers of male and female offspring that were produced.

## Results

Shuker et al. examined how patch partner relatedness affected offspring sex ratios. The main question of interest in the study was whether related females (those in treatments A and B combined) produced a more female-biased sex ratio than did unrelated females (those in treatment C). However, general linear models revealed that relatedness had no effect on the brood sex ratios (sex ratio of related:  $0.260 \pm 0.007$ ; sex ratio of unrelated:  $0.236 \pm 0.013$ ;  $F_{1,339} = 2.587$ , P = 0.109). The results from Shuker et al. (2004) are presented in Figure 4.4.

We reanalyzed these data using a MANOVA to test for the effect of treatment on two response variables: the number of male offspring produced and the number of female offspring produced. If a MANOVA revealed a significant difference between



Figure 4.4. Data from Shuker et. al. 2004. Female sex ratio behavior and oviposition partner. Females were either with a female from the same strain (black bars: HV236; white bars: HV307) or were unrelated (gray bars: one HV236 female with one HV307 female). Additionally, females from the same strain were either raised on the same host (SH) or different hosts (DH). Error bars show standard errors.

groups with respect to a particular factor, we continued with univariate post-hoc analyses to determine which combinations of dependent variables were responsible for inter-group differences. Specifically, we tested for treatment effects on the dependent variables themselves (number of male offspring produced, number of female offspring produced) and on two combinations of the dependent variables (total number of offspring produced, sex ratio of the offspring). The sex ratios were arcsine-transformed for the analyses. All analyses were conducted using MATLAB 7.0. Means are presented  $\pm$  SE.

In both wasp strains, treatment had a significant effect on sex allocation behavior (Wilks'  $\Lambda_{2,338} = 0.915$ , P<0.0001; Figure 4.5). Since this MANOVA indicated a significant effect of treatment, we proceeded with additional MANOVAs that tested for differences between each pair of treatments (Scheiner and Gurevitch 2001). There was no significant difference between treatments A and B, indicating that wasps in treatments A and B did not differ in their sex allocation decisions (Wilks'  $\Lambda_{1,273} = 0.990$ , P=0.242). Thus, related wasps made similar reproductive decisions when ovipositing, independent of whether they were raised on the same or on a different host. Wasps in treatment C made different reproductive decisions than did wasps in either treatment A (Wilks'  $\Lambda_{1,199} = 0.882$ , P<0.0001) or treatment B (Wilks'  $\Lambda_{1,204} = 0.900$ , P<0.0001).

To test the hypothesis that related females make different sex allocation decisions when ovipositing together than do unrelated females, we compared the sex allocation decisions of females in treatment C with those of females in both strains across the combined treatments A and B. There was a significant effect of relatedness on sex allocation decisions (Wilks'  $\Lambda_{1,339}$  = 0.923, P<0.001; Figure 4.5). Unrelated females produced a significantly smaller combined clutch than did related females (combined



Figure 4.5. Wasp sex allocation behavior in relation to oviposition partner (mean  $\pm$  SE number of female and male offspring produced). Females were either with a female from the same strain or were unrelated (one HV236 female with one HV307 female). There was a significant effect of relatedness on sex allocation decisions (Wilks'  $\Lambda_{1,339} = 0.923$ , P<0.001).

clutch size of related: 111.386 ± 2.752; combined clutch size of unrelated: 78.773 ± 5.103;  $F_{1,339}$  = 28.123, P < 0.0001). This smaller combined clutch size of unrelated females was due to their lower production of both male and female offspring compared to the related females (males:  $F_{1,339}$  = 14.494, P < 0.001; females:  $F_{1,339}$  = 23.841, P < 0.0001). There was no significant difference in sex ratio between related and unrelated females (sex ratio of related: 0.260 ± 0.007; sex ratio of unrelated: 0.236 ± 0.013;  $F_{1,339}$  = 2.587, P = 0.109).

#### Discussion

The conclusions drawn from this study depend on whether the data are analyzed using a sex allocation or a sex ratio approach. Related and unrelated female wasps made different offspring sex allocation decisions. Related females produced more female and male offspring than unrelated females, and in total laid nearly 50 percent more eggs than unrelated females. Why this might be remains a puzzling biological pattern that is deserving of future work.. Yet these results raise the possibility that female *Nasonia vitripennis* wasps have the ability to assess kinship with oviposition partners.

In contrast, an earlier analysis of sex ratio decisions in this dataset led Shuker et al. (2004) to conclude that wasps cannot assess kinship. Their analysis assumed that sex ratios were the variable of interest. Indeed, the predictions of LMC were originally framed in a sex ratio context, as Hamilton (1967) assumed that females could vary sex ratio but not clutch size. Based on the results of this study, future LMC theorists should build models incorporating the ability of females to influence both the sex and the number of their offspring. More generally, this reanalysis highlights the importance of

considering both sex ratio and clutch size as ways by which parents can adjust their sex allocation.

## Conclusions

Sex allocation and sex ratio are not identical terms. Whereas sex allocation theory examines an organism's total joint investment in male and female offspring [males, females], sex ratio theory collapses these two dimensions of information into the sole dimension of sex ratio [males/(males + females)]. In the examples we presented above, tests of sex ratios failed to reveal a significant effect of experimental treatment on the reproductive decisions of honeybee queens and female parasitoid wasps. However, tests of sex allocation revealed significant and important influences of experimental treatment on reproductive decisions. The examples in this paper illustrate that the framing of questions asked by biologists can greatly affect the conclusions they draw from their studies.

The examples in this paper focused on sex allocation decisions by haplodiploid insects. In haplodiploid species, haploid (unfertilized) eggs develop into males and diploid (fertilized) eggs develop into females. Females in these species can therefore precisely control their egg sex allocation by controlling whether or not they fertilize their eggs (Ratnieks and Keller 1998). Recent evidence suggests the ability to adaptively adjust offspring sex allocation isn't limited to haplodiploid species, but potentially also occurs in birds and other vertebrates with chromosomal sex determination (West and Sheldon 2002; Holand et al. 2006). Therefore, females across a wide variety of taxa are

able to adjust their offspring sex allocation.

As for how females actually adjust offspring sex allocation, there are several potential strategies. For example, to produce a greater investment in male offspring, a female might increase her offspring sex ratio, increase her clutch size, or use some combination of these strategies (Figure 4.6). Each of these strategies makes implicit assumptions about the biological constraints faced by the organism (West and Sheldon 2002). If females are constrained by the number of offspring they can produce, they might increase their investment in males by increasing their offspring sex ratio. This assumption was used by Hamilton (1967) when he formulated LMC theory. If females instead are constrained in altering the sex of their offspring, they might increase their investment in males by increasing their offspring, they might increase their investment in the sex of their offspring, they might increase their investment in males by increasing their offspring, they might increase their investment in the sex of their offspring, they might increase their investment in the sex of their offspring, they might increase their investment in the sex of their offspring, they might increase their investment in the sex of their offspring, they might increase their investment in the sex of their offspring, they might increase their investment in the sex of their offspring.

A subtle shift in both sex ratio and clutch size can affect the number of males produced, but might not be captured by traditional statistical methods that test for the effects of treatment on sex ratio or clutch size. An excellent example of this can be found in the wasp dataset from above. Within the HV307 strain of wasps, females from the same or different hosts did not demonstrate statistically significant differences in either their offspring sex ratio ( $F_{1,130} = 0.571$ , P = 0.451) or clutch size ( $F_{1,130} = 0.976$ , P = 0.325). However, by using the number of males and females in a multivariate analysis of variance (MANOVA), we found that these wasps made different sex allocation decisions (MANOVA, Wilks'  $\Lambda_{1,141} = 0.955$ , P = 0.052; Figure 4.7). This difference was due to a significant difference in the number of male offspring ( $F_{1,130} = 5.324$ , P = 0.023). This example suggests that by treating the numbers of male and female offspring as dependent



Figure 4.6. Three ways that a female can increase (i.e., double) her production of males. A female might a) alter her sex ratio (dashed line), b) alter her clutch size (dotted line), or o) slightly alter both sex ratio and clutch size (dashed-dotted line). By any one of these strategies, a female can double her production of males (i.e. switch from producing 25 males to producing 50 males). Each pie represents a female's investment in male offspring (gray areas) and female offspring (white areas).



Figure 4.7. Wasp sex allocation behavior within the strain HV307 (mean  $\pm$  SE number of female and male offspring produced). The wasp sisters were either from the same host or from different hosts. The slopes of the dashed lines indicate the mean sex ratio (males/total) for each group (long dashes: same host; short dashes: different host).

variables, it is possible to detect biological patterns that might not be captured by traditional analyses that use either sex ratio or clutch size as the dependent variable.

Many researchers use sex ratios as their variable of interest because it is not feasible to collect data on the total numbers of males and females in a clutch. Indeed, in species that produce clutches consisting of thousands of eggs, it is often most practical to sample. Sampling usually involves recording the number of males and females of some predetermined number of offspring, which allows researchers to estimate sex ratios but not on the number of males and females in the entire clutch. Based on the findings in this paper, we suggest that researchers attempt to collect, whenever possible, the total number of males and females produced in a clutch. By doing so, they might be able to draw more insightful conclusions about sex allocation than they would by focusing on sex ratios.

We suggest that an exclusive focus on sex ratios can lead to a number of potential problems. First, a focus on sex ratios makes some limiting assumptions about the biology of the species and the constraints that they face. Indeed, species are believed to display a great variation in the degree to which females can adjust their offspring sex ratio (West et al. 2002). Second, a focus on sex ratios leads to statistical tests that might overlook other important components of sex allocation behavior, such as clutch size. Many tests statistically control for differences in clutch sizes rather than treat clutch size as a potentially important variable (Hardy 2002). Third, a focus on sex ratios can fundamentally change the conclusions that are drawn from the study, as demonstrated in the examples in this paper.

In conclusion, biologists might gain more insightful interpretations of their system of study by focusing on two-dimensional sex allocation decisions rather than one-

dimensional sex ratio decisions. Sex allocation theory provides a powerful way to determine the influence of environmental factors on animal behavior (West et al. 2000). We encourage researchers in behavioral ecology to examine sex allocation decisions, of which sex ratios are one, but not the sole, component.

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