EXPLORING WINNER EFFECTS IN QUEENS OF THE WESTERN HONEY BEE

BY

KARI JACKSON

THESIS

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Master's Committee:

    Professor Gene Robinson, Chair, Director of Research
    Associate Professor Alison Bell
    Professor Andy Suarez

**ABSTRACT**


Animals often engage in intraspecific conflict to increase their access to resources, and

for social species, to improve their rank within the society. Differences in intrinsic fighting

ability contribute to differences in contest success. However, prior experience can also contribute

to the outcome of conspecific competition. Success in a previous encounter can increase

aggressiveness and raises an individual's probability of winning future contests, a phenomenon

known as the winner effect. Likewise, a prior loss can promote docility and reduce the

probability of future wins, known as the loser effect. While the physiological mechanisms

underlying these effects are largely unknown, research on vertebrates and invertebrates supports

that winner and loser effects are also influential in the formation of dominance hierarchies.

Theoretical and empirical research suggests that a winner effect can only exist in the

presence of a loser effect. However, recent evidence suggests that this is not always the case.

This finding prompted the present study, which explores the presence of a winner effect in virgin

honey bee queens *(Apis mellifera)*.

Following the death or departure of the previous queen in a swarm, young virgin queens

engage in fatal contests for the opportunity to become the new reproductive individual in the

colony. To test for winner effects, I reared virgin queens and staged duels between pairs in Petri

dishes. The duels occurred between either two age-matched queens or between a younger and

older queen. Following this, the winner of the contest was pitted against another age-matched

queen with no fighting experience. The results indicated that prior winners did not experience an

increase in winning probability, and thus a winner effect was not detected. This may be

attributed to the lethal nature of the contests, which can promote high-risk injury and reduce the

importance of prior experience. The study also led to observation of novel behaviors including proboscis pairing between opponents. Finally, this study provides novel insight into the behavioral displays (antagonistic and non-antagonistic) produced by virgin queens whose outcomes determine the genetic composition of their entire colony.

# ACKNOWLEDGMENTS

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# INTRODUCTION

Aggression and conflict are common features of many animal populations. Individuals and groups often compete for access to resources, such as food, mates, and territory nest sites (Hsu et al., 2006) and can resort to aggressive tactics to acquire them. Shared requirements for growth, reproduction, and survival result in the intensity of intraspecific competition being greater than that of interspecific competition (e.g. Begon, 2010, Connell, 1983, and Anderson & Whitman, 2015). The outcomes of these aggressive encounters can have long-lasting influences on the fitness of individuals as well as the social structure of their groups (Laskowski et al., 2016).

Examination of competitive encounters has historically focused on how differences in intrinsic qualities of opponents (i.e. size, weight, age, weaponry) contribute to the final outcome (Hsu et al., 2006). More recently, attention has been directed toward the influence of experience on contests between conspecifics. Scientists recognize that most competing organisms possess an intrinsic fighting ability known as their resource holding potential or resource holding power (RHP) (Parker 1974). Following combat with a conspecific, an organism's perception of its RHP can be modified. This change can increase or decrease the probability of the organism "winning" future encounters. The cumulative effects of these physiological and behavioral modifications are known as "winner" and "loser effects, and have been well documented in mammals (Oyegbile & Marler, 2005), reptiles (Garcia et.al., 2014) , fish (Bakker et al., 1989), birds (Drummond & Canales, 1998), and invertebrates (Teseo et al., 2016).

Winner and loser effects are defined in two ways. Chase et.al (1994) defined the loser effect as a "*tendency for an animal losing a prior contest to lose a subsequent one with an*

*otherwise equally matched second individual*" with the inverse defining the winner effect. It

should be noted that the equally matched second individual should be naïve and have no prior

fighting experience when the conspecific encounters the prior winner or loser. Winner and loser

effects are also defined as having "*a higher tendency to behave aggressively in the current*

*conflict, while losers in previous conflicts may act more submissively*" (Van Dorn et al., 2003).

The definitions do not drastically differ and their requirements often overlap in numerous

studies. Increases in aggression/submissiveness are related to winning/losing probabilities.

However, the former emphasizes the outcome of intraspecific encounters while the latter is

concerned with a mechanistic explanation. Additionally, level of aggressiveness/submissiveness

does not always correlate to competition success. In my thesis work, I use Chase's definitions of

winner and loser effects.


**Actual vs. Perceived RHP Modification**

Experience effects can influence an organism's fighting behavior by either modifying its

RHP or its perception of its RHP (Hsu et al., 2006). Modification of the RHP implies that

following a contest, a contestant will undergo physiological alterations that directly increases or

reduces its intrinsic fighting ability.

Support for this comes from Kasumovic et al. (2010), who studied experience effects in

male jumping spiders (*Phidippus clarus*). Males that won their first contests escalated

subsequent conflicts at faster rates and fought longer. Males that lost their first contest also

escalated subsequent conflicts at reduced rates compared to winners, while their contest duration

shortened. RHP perception alone is not thought to influence contest duration; RHP perception

and actual RHP can both be modified, so contest duration change does not rule out that RHP

perception changes are also occurring. However, Kasumovic et al. (2010) did not address the mechanisms responsible for RHP modification in jumping spiders.

Garcia et al. (2014) examined the possible relationship between future contest outcomes and status-dependent changes in metabolic physiology in green anole lizards (*Anolis carolinensis*). Metabolic physiology was recognized as an important element for the RHP of numerous species. Individuals will proceed with or retreat from a conflict depending on energy expenditure and lactic acid accumulation. No significant correlation was found between metabolic physiology and secondary contest outcome, and thus winner and loser effects were not associated with actual RHP modification. Many other studies have drawn the same conclusions, demonstrating that when contests escalated to more costly physical interactions, the value of conspecific fighting experience declined and the conflict was settled by the individual with the greater RHP (reviewed by Hsu et al., 2006).

**Combatant Assessment Strategies**

As combatants prepare to engage, they may employ one of two assessment strategies to measure asymmetries in fighting ability. If an individual possesses private information of its RHP as well as public information of its opponent's RHP (the average distribution of RHP for the population + cues/signals that relate to their specific opponent) it can utilize a self-assessment strategy (Mesterton-Gibbons & Heap, 2014). An individual can use this information to compare RHP differences and determine whether it is beneficial to engage and persist in a contest or withdraw and preserve its health. A pitfall of this technique is that reliance on the average RHP can lead to potential assessment errors in which a focal individual over-estimates (expending

excessive energy to subdue or retreats when they should have continued) or under-estimates its opponent's ability (under-utilizing energy stores or continues when retreat is the better option).

A more accurate method is the mutual-assessment strategy (social-cue) in which the individual collects private information of its opponent's RHP and compares it with its own. This specific information is typically acquired during physical displays that precede combat (Whitehouse, 1997). One can analyze an opponent for signs of stress (possibly inflicted during a prior contest) or production of chemical cues (hormones) that arise following intense combat (Rutte et al., 2006). In one study, rusty crayfish (*Orconectes rusticus)* that were continuously exposed to odors produced by dominant conspecifics were less likely to initiate and win future fights while those exposed to odors of subordinates were more likely to win (Bergman & Moore, 2005).

Despite being the more adaptive of the two strategies, mutual-assessment also has costs. The risks of obtaining information of another's RHP can outweigh its benefits if the opponent attacks during assessment. There may also be physiological costs for acquiring and maintaining accurate assessment abilities. Of greatest concern, attempts at "learning" the RHP of one's opponent may leave individuals vulnerable to attack from nearby predators (Mesterton-Gibbons & Heap, 2014).

The choice of assessment strategies may depend on the condition of conflict (Mesterton-Gibbons & Heap, 2014). The mangrove killilish (*Kryptolebias marmoratus*) initially adopts the mutual-assessment technique to determine whether escalation is beneficial (Hsu et al., 2011). The more similar in size two opponents were, the more likely the conflict was to escalate. However, as the contests escalated to mutual attacks, the fish used self-assessment (based on the escalated contest duration correlated with loser's size but no relationship developed with the

winner's size). Modeling work suggests that mutual-assessment benefits the stronger members of a population and is detrimental to weaker ones if contests are more costly then beneficial. If the benefits of the conflict outweigh their costs, both groups benefit although stronger members potentially can win any fight and mutual-assessment would provide a minute advantage (Mesterton-Gibbons & Heap, 2014).

Other considerations for assessment preference may depend on current availability of the resource. If, for example, an individual is looking for a mating partner in the earlier period of a breeding season, it is more likely to use a mutual-assessment strategy against its competition if there are multiple mating opportunities (i.e., the resource has moderate value). Individuals can take more calculated risks in directly assessing their opponents because if they fail in their attempt, they can pursue more opportunities. However, if their mating options are considerably reduced (resource has greater value), they will choose the self-assessment strategy because they can no longer afford the risk of direct opponent assessment.

Despite self and mutual-assessment serving as the primary assessment techniques, there are instances in which it is unclear whether either or neither are employed (Hsu et al., 2006). Morell et al. (2005) observed whether self-assessment or mutual-assessment predicted contest duration in male Australian fiddler crabs (*Uca mjoebergi*). Under mutual-assessment, it is expected that contest duration will be determined by the relative size of the crabs and will decrease if relative difference in body sizes increases. However, self-assessment mechanisms predict that duration is dependent on the crab with the lower RHP threshold and the contest ceases when the weaker individual retreats. In Morell et al. (2005), contest duration was positively correlated with increased size of the loser and negatively correlated with increased size of the winner, implying that fighting duration increases when opponents are of similar size.

However, the loser's size had a stronger influence and was the best predictor for contest duration. This suggests that an individual's absolute RHP determines contest duration (self-assessment). Based on these results, it appears that the crabs use a strategy that incorporates aspects of self and mutual-assessment but operates as a separate assessment technique. It should be noted that the experiments in Morell et al. (2005) were designed to prevent the onset of winner and loser effects by staging contests between males who won their previous fights.

## Winner Effect vs. Loser Effect

Winner and loser effects may not be controlled by the same physiological processes. Studies on the fruit fly (*Drosophila melanogaster*) (Trannoy, et al., 2016) and cichlid fish (*Oreochromis mossambicus*) (Oliveira et al., 2009) provide evidence that winner and loser effects are driven by closely-related but independent mechanisms. In *O. mossambicus*, androgen fluctuations mediated winner effects but had no influence on loser effects. *De novo* protein synthesis was only associated with a loser effect but not a winner effect in *D. melanogaster* (Trannoy, et al).

In both vertebrate and invertebrate species, it is commonly observed that the loser effect persists longer than the winner effect (Hsu et al, 2006); rarely do both effects persist for equal periods of time (Hsu &Wolf, 1999). Loser effects may be more prominent because of differences in costs accrued. Mesterton-Gibbons (1999) predicted that after a contest, opponents reassess their RHP perception and accumulate a cost proportional to the amount they overestimate their RHP. In subsequent contests, overestimation of one's RHP will become more costly and/or fatal than underestimation and thus is more likely to be extinguished. This may explain why several studies produced a loser effect without a winner effect but there were no studies that produced a

winner effect without a loser effect. However, Goubault and Decuignie`re (2012) showed that females of the parasitoid wasp *Eupelmus vuilleti* only experience a winner effect when they compete for a host. This finding has opened the opportunity to explore experience effects in species that have been previously disqualified due to aspects of their natural history that eliminate the possibility of a loser effect, such as the western honey bee (*Apis melifera*).

**Experience Effects and Dominance Hierarchies**

Examining experience effects can provide insight to the formation of linear dominance hierarchies in nature (Mesterton-Gibbons et. al, 2016). In dominance hierarchies, individuals in a population develop different hierarchical ranks in terms of accessibility to resources; higher-ranking members typically have greater access compared to lower ranking members. Despite the uneven distribution of assets, linear hierarchies are adaptive in promoting higher resource accumulation and protection from predation in some ecological settings (Hewitt et al., 2009). Landau (1951a, b) produced models that examined how intrinsic and extrinsic factors lead to the formation of linear hierarchies. It was only when both types of factors were included in the models that they produced hierarchies consistent with those found in nature (Landau 1951a, 1951b). Consistent with this, the development of dominance hierarchies is attributed to an organism's RHP as related to its size and fighting ability, and extrinsic attributes, which include previous contest experience (Dugatkin, 1997).

More recent models have explored the consequences of group members perceiving their RHP and the collective RHP of everyone else (Dugatkin, 1997). After a contest, an individual's perception of its RHP was modified (decreases or increases) while its perception of the others' RHP remained stable. Isolating experience effects led to different results. The presence of only

the winner effect led to strict linear hierarchies with explicit position rankings. However, the loser effect produced one dominant individual and a collection of others with unclear positions. When the model enabled conspecifics to assess the RHP of opponents and how it changed over time, strict hierarchies formed in the presence of winner and loser effects (independently or combined) (Dugatkin & Dugatkin, 2007). Ultimately, experience effects may be adaptive for dominance hierarchies because subordinates under the influence of loser effects reduce fighting costs whereas dominant figures under the influence of winner effects preserve energy that would have been used for fighting, therefore minimizing continuous tests of RHP while stabilizing interactions (Hock & Huber, 2008).

Empirical support for experience effects undermining dominance hierarchies comes from research on coyotes*(Canis latrans)*, green swordtail fish (*Xiphophorus hellerii*), amazon clonal fish (*Poecilia Formosa*), and cichlid fish *(Metriaclima zebra)* (e.g. Bekoff & Dugatkin, 2000, Dugatkin & Druen, 2004, Laskowski et al., 2016, Chase et al., 2002). In green swordtail fish, dominance hierarchies were established by pairing focal males with larger males (expected to produce a loser effect), smaller males (expected to produce a winner effect) or with no pairing (control). After the winner, loser, and control pairings were produced, they were grouped together and observed for 3-7 days for the emergence of a hierarchy. Of the six possible structural combinations, the most frequently documented was the winner fish having the highest rank, the control fish having the middle rank, and the loser fish having the lowest rank. These and other findings demonstrate that long-standing rankings in a hierarchy can be dictated by experience in the formative periods of an organism.

**Basis for Winner and Loser Effects**

Despite the considerable amount of data documenting experience effects, there is limited information to explain how they arise. Currently, there is no comprehensive explanation for the proximate and ultimate causes of winner and loser effects. One possibility is that experience effects are by-products of status-dependent changes in physiology (e.g., changes in hormone production) during a contest (Hsu et al., 2011). Changes in androgen and corticosteroid release are the best documented (Hsu et al., 2006). Many vertebrate studies have revealed that the winner effect is correlated with elevated levels of androgen and the loser effect is correlated with elevated levels of corticosteroid. However, there are no consistent studies that directly link a winning experience with elevated androgen under natural conditions (Hsu et al., 2006).

One explanation is experience effects result from changes in levels of hormones: such as androgen, corticosteroid, and serotonin (Hsu et al., 2006). After a contest, winners and losers may have pronounced changes in hormone production of these chemicals, which can increase or decrease their aggression in subsequent competitions. However, inherent physiological differences between vertebrates and invertebrates limit hormones as proximate causes for winner and loser effects. Serotonin, for example, is associated with decreased aggression for vertebrates but prolongs aggressive behavior in invertebrates (e.g., Hsu et al., 2006, Rutte et al., 2006).

Duration of an effect is also not directly linked to the duration of elevated hormone levels. Male copperheads (*Agkistrodon contortrix*) that lose a contest exhibit a loser effect for seven days despite corticosterone levels returning to baseline after an hour of competition (Rutte et al., 2006). It is unlikely that winner and loser effects are simply by-products of physiological processes.

Experience effects may involve a reorganization of neural pathways that lead to learning (Hsu et al., 2011). Experience causes changes in steroid hormone concentrations which in turn lead to enhanced synaptic strengths or changes in neurochemicals. This may alter perceived fighting ability following losses and wins. Changes in neuromodulators, neurotransmission, and gene expression patterns following a contest may also contribute to winner effects (Hsu et al., 2011). It is likely that a combination of all these factors is necessary for experience effects to form.

Evolutionary explanations of winner and loser effects are based on the notion that organisms originally competed in triad round-robin competitions (Fawcett &Johnstone, 2010). While the competitors had no idea of their RHP, their perception was driven by their fighting outcomes. However, opponents acquired costs proportional to how much they overestimated their abilities, regardless of the outcome. Winner and loser effects are predicted to emerge under these circumstances. Van Dorn et al. (2003) produced models in which conspecifics interacted in a hawk-dove game and they only retained memory from their most recent interaction with each opponent. Multiple simulations yielded evolutionary equilibria when winner and loser effects were factored. This suggests that experience effects serve as a means to settle disputes and stabilize the community.

**Western Honey Bee**

The Western honey bee (*Apis mellifera*) is one of the most important species of insects on the planet. Honey bees feed on pollen and nectar produced by flowers (Winston, 1987). Subsequently, the bee's pollination services are of great ecological and economic significance, responsible for over 90 commercially produced crops in North America (Status of Pollinators in

North America, 2007) as well as crop production worth over $14 billion dollars in the United States (USDA, 2016).

The honey bee has become a model for the study of social behavior, particularly eusociality. Eusociality is characterized by overlapping generations, cooperative brood care, and division of labor into non-reproductive and reproductive groups (Wilson & Holldobler, 2005). The honey bee has three distinct types of individuals: drones, workers, and queens. Drones (males) are responsible for inseminating queens, which leads to their death as a result of rupturing of the genital apparatus (Winston, 1987). Because they do not provide other services to the colony, they are removed by workers if resources are limited. Workers (females) compose the majority of bees within honey bee colonies and are responsible for numerous tasks, ranging from feeding young larvae to protecting the nest from potential threats. Adult worker honey bees display temporal polytheism: workers will gradually cease to perform one task and perform another in a predictable age-related pattern (Johnson, 2010). The queen's primary function is egg laying and she can produce up to one million eggs in a lifetime (Winston, 1987). Early in life she will take part in mating flights with drones and store sperm within her spermatheca for years.

Despite workers and queens sharing identical genotypes, they express different phenotypes as a result of their diet during development. Worker larvae are fed mandibular gland secretions, hypopharyngeal gland secretions, and pollen in a 2:9:3 ratio (Winston, 1987). Queen larvae are fed "royal jelly", which involves a1:1 ratio of mandibular and hypopharyngeal secretions, and are fed in higher quantities than are worker larvae. The different diets lead to differential epigenetic modification including DNA methylation (Pierce, 2014) and histone modification (Spannhoff, et.al, 2011). Kucharski, et al. (2008) experimentally silenced the expression of the methyl-adding DMNT3 enzyme in newly hatched female larvae. This led to the

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development of bees with queen characteristics (i.e. developed ovaries and larger size) that normally emerge from the consumption of royal jelly. This suggests that royal jelly may induce genes that promote morphological and physiological rearrangements in honey bees.

**Swarming and Queen Production**

Honey bees undergo colony reproduction, with the reproductive queen departing with over half of the colony's workers to form a separate colony (Tarpy et al., 2003). Known as swarming, this process takes place during mid-spring and early summer in temperate habitats (Winston, 1987). In the weeks prior to the first swarm, workers produce specialized cells for young larvae to develop into virgin queens. Following the primary swarm, queens emerge from their cells and produce high-pitched sounds by pressing their thorax to the comb ("pipping"). This pipping is thought to inhibit worker aggression towards the younger queen (Gilley, 2001) or advertise fighting ability (Schneider et al., 2001). Virgin queens will either stay in the colony to fight and kill their sister queens or depart with a smaller number of workers in an after-swarm (Tarpy and Meyer, 2009).

There is only one laying queen in a honey bee colony. Elimination of the supernumerary queens occurs either by a recently emerged adult virgin queen destroying the cell containing a developing rival or by direct fights known as "duels." Cell destruction entails a queen chewing a 3–5 mm diameter hole in the side wall of queen cells and stinging the developing larvae (Gilley & Tarpy, 2005). Queens are able to discriminate cells and target those that contain the oldest larvae, as they represent their biggest threats. If a queen is able to eliminate all rivals in this manner, pre-emptively, she inherits the colony. Otherwise, she will come in contact with other recently emerged queens and must then duel.

It remains unclear if success in these duels is associated with the sequential order of emergence of virgin queens in the nest. Gilley & Tarpy (2005) reported that the queens that emerged last or next to last inherited their colony following the queen duels. Schneider et.al (2001) reported that being the first queen to emerge was not strongly associated with survival. However, Schneider & DeGrandi-Hoffman (2007) determined that order of emergence significantly influences colony inheritance in colonies that did not produce after-swarms: 87.5% of the queens that inherited their colony were the first to emerge. These queens eliminated their rivals via pre-emergence destruction and queen duels. This may imply that under these conditions, having more interactions with potential competitors confers an advantage for slightly older virgin queens. It is possible however that prior residency within the hive is advantageous to older queens and improves their probability of defeating younger opponents. A residency effect in conspecific contests has been demonstrated in social animals (e.g., Fuxjager et al., 2009, Fuxjager &Marler, 2010).

The high cost associated with queen duels would suggest that leaving in an after-swarm between duels is the better option. However, virgin queens and workers leaving in after-swarms have a low overwintering survival rate in general, especially in temperate climates, because after-swarms are smaller than the primary swarm (e.g. Winston, 1987, Gilley &Tarpy, 2005). Of the three options described, elimination by queen duel is the most common fate of virgin honey bee queens (Gilley & Tarpy, 2005).

**Dueling Behavior Description**

Young virgin queens can tolerate each other for several hours to days (Winston, 1987) and can come within 1cm without reacting to each other (Gilley & Tarpy, 2005). However, when

contact is made, virgin queens grapple and attempt to sting each other (Gilley, 2001). The stinging sequence consists of 1) the queen positioning herself towards her opponent's abdominal tergites, 2) grabbing/positioning her conspecific with her legs, and 3) bending the abdomen to sting her opponent's thorax (Pflugfelder and Koeniger, 2003). It has been speculated that there is a releaser pheromone for stinging behavior located under the queen's tergite gland but this has not been demonstrated (Pflugfelder and Koeniger, 2003). Compared to workers, queens have smaller barbs on their stingers but are capable of retraction and greater venom production (Winston, 1987).

Queen duels are dyadic and fatal, with the defeated virgin queen succumbing to her injuries. Duels can last from several seconds to hours (Tarpy & Fletcher, 1998) and queen elimination activities (also known as "tournaments") lasts for up to a week (Gilley & Tarpy, 2005).

**Impact of Colony Workers on Virgin Queen Survivorship**

Adult worker bees play a critical role in queen elimination, both before and after the emergence of virgin queens. Workers can "imprison" mature queens within their cells while providing extra food through slits (Gilley, 2001). It is possible that this reduces the onset of queen duels and gives the virgin queens a better chance of departing in an after-swarm. Workers also will destroy queen cells damaged by rival queens and dispose of the queen pupae inside (Gilly & Tarpy, 2005). As the virgin queen assesses a rival queen cell, she may be harassed by workers who will grab, clamp and chase her away (Gilley, 2001). These worker displays, while not fatal, can leave queens vulnerable to attack by rival conspecifics (Gilley & Tarpy, 2005).

Workers may also produce a vibration signal (vibrating the body dorsoventrally for 1–2 seconds) while clinging to queen cells or virgin queens. Despite no association between vibration signal rate and emergence success, worker vibration on virgin queens was positively associated with increasing piping, longer survival rates, and greater fighting success (Schneider et al., 2001). Vibration may be a means for workers to influence queen aggression but because the data are correlational, this cannot be determined conclusively.

Worker behaviors towards virgin queens may be influenced by the genetic relatedness of the individuals involved. The haploid-diploid nature of honey bees and the multiple mating by queens contributes to workers having sisters of low (half-sisters) and high (full-sisters) genetic relatedness (Gilley, 2003). This can lead to an ecological conundrum of whether workers should improve their inclusive fitness by favoring queens who are more genetically similar to them or improve the group fitness of their colony by favoring queens of higher fecundity potential (Gilley et al., 2003). Despite this potential conflict of interest, nepotism is rarely displayed by adult worker bees (e.g. Breed et al. 1994, Visscher 1998) who instead collectively rear queens best suited for the colony (e.g. Chaˆline, et al., 2004, Tarpy et al., 2004). There is also no evidence that workers more frequently harass queens that are less related to them during queen duels (Gilley, 2003). While Tarpy and Fletcher (1998) demonstrated that virgin queens that are more related to workers had a higher probability of survivorship in their colonies, they compared queens that were genetically related to the workers to those that were unrelated.

**My Study**

The purpose of this study was to determine if experience effects contribute to the outcome of queen duels. More specifically, it was to observe if the dueling behavior of virgin

honey bee queens is consistent with the prediction of a winner effect: does winning the first contest increase a queen's probability of winning subsequent encounters? The fatal nature of these competitions eliminates the possibility of a loser effect. However, it is possible to have a winner effect without a loser effect (Goubault and Decuignie`re, 2012).

As the primary reproductive individual in a eusocial system, the queen contributes tremendously to the genetic structure of her colony. In essence, the genetic composition of a colony is the extended phenotype of a honey bee queen and her mates (Tarpy et al., 2011). Colony fitness and survival strongly depend on queen reproductive fitness (Long et al., 2016). Inherent variation in reproductive fitness exists among queens, with individual differences in fecundity, longevity, and fertility (Tarpy et al., 2011). If a winner effect that is independent of actual RHP arises during queen duels, then queens with lower reproductive fitness can inherit colonies, permanently altering their genetic structures and potentially impacting colony health and stability.

There are several common patterns among taxa that exhibit experience effects. A critical component is that conspecifics compete in highly aggressive contests to obtain a desired resource and/or achieve higher rank in their society (Hsu et al., 2006). This is true of virgin queens, who compete for the opportunity to become the sole reproductive unit of their colony. As mentioned earlier, a colony can produce more than 20 virgin queens and elimination duels last nor more than seven days. This suggests that a queen who has won a duel will encounter at least one more queen during this period. In many species, one encounter post win/loss is enough to observe a winner or loser effect (Hsu et al., 2006).

Previous observations have suggested that a queen will only encounter one opponent inside the honey bee nest (Gilley, 2001). However, the average numbers of queens that emerge

per nest in these studies (2-4) are much lower that what has been previously reported in the literature. As the number of emerging virgin queens increases in a hive, the rate of interactions between these opponents increases (Gilley & Tarpy, 2005). Additionally, under the circumstances in which an after-swarm does not occur, the first virgin queen to emerge typically inherits her nest and kills proportionally higher number of rivals than her conspecifics (Schneider & DeGrandi-Hoffman, 2007). This would support the notion that having more experience is beneficial to competing virgin queens.

The value of prior contest information is negatively correlated with frequency of social encounters. Hsu et al. (2006) posits that for species that engage in aggressive encounters at higher frequencies, conspecifics will more likely use information from the most recent encounter than information collected from past encounters, reducing the longevity of experience effects. Likewise, experience effects will persist longer when conspecific interactions are infrequent. The asynchronous nature of queen emergence (Schneider et.al 2001) and queen's unwillingness to immediately engage in conflict (Gilley & Tarpy, 2005) suggests that contest interactions are uncommon and that prior experience would support the emergence of the winner effect.

Finally, while a dominance hierarchy is lacking among honey bee queens, winner and loser effects have been demonstrated in species lacking this social system (e.g., Trannoy, et al., 2016, Rillich & Stevenson, 2011, Hsu & Wolf, 1999, Wilgenburg et al., 2010). Thus, a winner effect is predicted based on virgin queen bee biology.

I hypothesize that honey bee queens retain and utilize prior experience during their combats and that the outcome of queen duels is not solely dependent on asymmetry in fighting ability. Furthermore, I hypothesize that the winner effect forms in the queen duels. I tested these hypotheses by placing two virgin queens in a fighting arena, recorded their behaviors, and

recording the winner of the contest. After a period of rest, the winning queen fought another

queen who had no prior fighting experience. Once again, their behaviors and the winner were

documented. This methodology was used to determine the emergence of a winner effect in the

bees.

# MATERIAL AND METHODS

## Experimental Setting and Bee Colonies

Experiments were conducted during the summer of 2016 at the University of Illinois Bee Research Facility, Urbana, Illinois. Bees were collected from colonies maintained according to standard commercial methods. Four different colonies (headed by naturally mated queens) were used to produce 260 virgin queen cells. Of these cells, 114 virgin queens successfully emerged and were used in 38 behavioral assays (3 queens per assay).

## Queen Rearing

Virgin honey bee queens were produced using standard commercial methods (Stahlman, 2013). Several days after the waxen queen "cells" were capped by adult workers, the cells were removed from the "queen builder" colonies and transported to an incubator room (32 °C ± 0.5 °C; ~60% relative humidity) for experimentation. The incubator room mimics the internal temperature and humidity maintained in natural honey bee colonies.

Each capped cell was placed at the edge of a Petri dish (8.6cm) with an opening made so that queen could enter inside after she emerged. The dish also contained a 1.7mL tube of honey, a 1.3g pollen ball (composed of honey bee pollen, honey and 50% sucrose water), a ~8.5cm wax surface that mimics honeycomb cells, and four newly emerged adult workers from a different colony to feed the queen. While these workers were derived from a different colony, they adapt immediately to the first queen they interact with (Winston 1987). Following queen emergence, the empty cells were removed and replaced with a plastic vial of 50% sucrose water. Each queen

was marked with a colored number plastic tag (manufactured by the Bee Research Institute at Dol, Czech Republic) for individual identification prior to experimentation.

**Experimental Design**

There are two experimental protocols for testing experience effects of competition: self-selection and random selection (Hsu et al., 2006). This study used both, but due to experimental limitations, emphasis was placed on self-selection (102 of 114 virgin queens were tested with self-selection). See Figure 1 for experimental configuration.

*Self-Selection.* To test winner effects, focal queens were aged to four days and placed in separate plastic vial tubes (5mL) that were coated in black ink on the outside. I was blind to the identity of the queens in their tubes. I randomly grouped the bees into units of three queens and randomly selected two to duel in the first encounter. Members of the selected pair were simultaneously placed in a Petri dish fighting arena (same dimensions as queen home dish) while the other four-day-old queen was placed in a separate Petri dish. If a queen defeated her opponent, the defeated opponent was removed and the winner queen was left undisturbed for either 15 or 50 minutes before being paired with her second opponent. The two queens fought and the winner of this duel was recorded. Of the 38 assays, 34 employed the self-selection protocol.

*Random Selection.* For this protocol, four-day-old virgin queens were paired with one-day-old virgin queens. This was done because Tarpy et al. (2000) demonstrated that age is a stronger and more consistent predictor of fighting ability than size, with older queens consistently outcompeting younger queens. Additionally, my preliminary observations revealed that older queens easily defeated younger opponents. The advantage older queens may have over

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younger queens may result from harder cuticle or greater pheromone production (Tarepy et al., 2000), but this not been explored.

The older queens were placed in black coated plastic vial tubes while the younger queens were placed in unmodified, transparent vial tubes. As with the self-selection assays, queens were randomly grouped into three-queen units. However, unlike for the self-selection assays, each unit contained 1 one-day-old queen and 2 four-day-old queens. One of the 2 four-day-old queens was randomly chosen to become a winner and so was paired with the one-day-old queen. They were placed in a Petri dish fighting arena while the other four-day-old was placed in a separate Petri dish. After the four-day-old queen defeated her younger opponent (which she did in all assays), the defeated opponent was removed and the winner queen was left undisturbed for either 15 or 50 minutes before being paired with her second opponent. The two queens fought and the winner of this duel was recorded. Of the 38 assays, 4 employed the random selection protocol.

In addition to the winner of each contest, the duel duration, the amount of time that passed before the queens initially engaged, and any other behavioral displays were documented. Queens only dueled with conspecifics that originated from the same hive and grafted in the same queen builder. If a queen did not fatally sting her opponent within 6 hours of her first duel and thus did not become a winner, the assay was terminated and both queens were returned to their original Petri Dish. The same applied to a winner queen who did not sting her opponent in her second duel.

Virgin queens for both selection protocols were observed under fluorescent light. Queen competition occurs within the hive which is normally dark. My preliminary observations however indicated that the dueling behaviors produced by queens under fluorescent light did not drastically differ from the dueling behaviors reported for queens dueling in Petri dishes under

dark conditions (Tarpy & Meyer, 2009).  When there were no observations, the incubator room was kept in dark to minimize potential long-term effects of fluorescent light exposure.

It is stated that when individuals are tested under random selection, the winner effect occurs if >50% of the winners win their subsequent duel (Hsu et al., 2006).  It is also stated that when individuals are tested under self-selection, the winner effect occurs if > 66% of the winners win their subsequent duel (e.g. Hsu et al., 2006, Begin et al., 1995). The increased expectancy for a winner effect to emerge under self-selection comes from Begin et al. (1995) who calculated that animals that win a contest against an equivalent conspecific have 66% probability of defeating an equivalent conspecific with no contest experience.

For my study, if a winner effect were detected in the 34 self-selection trials, then over 23 are predicted to produce virgin queens who won both duels. Likewise of the 4 self-selection trials, more than 2 are predicted to produce virgin queens who won both duels. This latter sample size is too small for statistical analysis however, so these results are only descriptive. I also hypothesized that a more statistically significant winner effect will be detected after a 15-minute interval as opposed to a 50-minute interval because the transient nature of winner effects in insects would lead to greater decay of the effect for the longer of the two intervals.

**Statistical Analyses**

One-tailed binomial tests were used to detect the presence of a winner effect. Two-tailed binomial tests also were used to test more generally for experience effects. It should be noted that SPSS automatically reports a binomial test as one-tailed if the test proportion is not equal to .50. To remedy this, I doubled the reported significance level (Williams, 2004).

Logistic regression explored whether the lengths of both duels predicted the outcome of the second duel. To determine how well the model performs with the inclusion of the predictor variables (duel lengths), the Omnibus Tests of Model Coefficients was performed. A sig. value of less than .05 supports that the model is more accurate than SPSS' original classification of predictions. Additionally, the Hosmer-Lemeshow Goodness of Fit Test was performed to determine how well the model fits the data. A sig. value greater than .05 indicates that the model fits the data.

Mann-Whitney Tests were used to determine if duel duration between virgin queens who won their first duel and lost their second duel and virgin queens who won both duels statistically diverged. A post-hoc power analysis was used to determine if there were a sufficient number of subjects to detect an actual effect.

All results were analyzed with SPSS 16.0 and R-Studio.

# RESULTS

Of the 38 queen duel assays (involving 114 virgin queens) conducted in the study, 35 assays produced winners in the first and second duels (105 queens) and three did not (Figure 2). In the three assays in which no winner was identified, virgin queens avoided each other and showed no aggression for several hours. Their movements declined as the duels continued (likely related to the extensive period without food), decreasing the likelihood of fighting behavior. When it was evident that they were not going to grapple/sting, they were returned to their dishes.

Thirty-one of the 35 remaining assays were used for the self-selection protocol (17 with a 15-minute interval between duels and 14 with a 50-minute interval). A total of 93 virgin queens were used for the tests that employed the self-selection protocol. Four of 35 assays used the random-selection protocol (2 with a 15-minute interval and 2 with a 50-minute interval). A total of 12 queens were used with the random-selection protocol.

The assays lengths of both duels for all queens are represented in a scatterplot (Figure 3). Additionally, the assay lengths of both duels for queens who only won the first duel (Figure 4) and queens who won both duels (Figure 5) are also represented in scatterplots.

For all 35 assays, there was no significant difference in the average length of the first duel between queens that only won their first duel and queens who won their first and second duel, (U=95.5, z=-.764, p=.445, r=0.13). Likewise, there was no significant difference in the length of the second duel between queens that only won their first duel and queens who won their first and second duel, (U=94.5, z=-.804, p=.422, r=.14)

Aggression between queens occurred within the first five minutes for 75% of the first duels and 66% of the second duels. For ~70% of the assays, grappling and stinging appeared

within seconds of queen introductions. With the exception of two duels, grappling/stinging occurred in all assays within 15 minutes after the trial began. After the queen fatally stung her opponent, she either avoided the dying/dead carcass of the other queen or examined/antennate her former combatant. The intensity of examination varied from visiting the body and moving to another location in a cyclical manner to allocating all attention to the loser. There was one instance of the winner queen grooming/licking her dying opponent.

In the 9 assays in which the focal queen fought for over 60 minutes in the first duel, only 2 queens also fought for over an hour in the second duel. The remaining 7 queens on average dueled for 12 minutes in the second duel. This is 5 times lower than the average length of the second duel (60 minutes) for queens who fought for under 60 minutes in the first duel. However there were no noticeable differences in rates of stinging and grappling between these two groups.

There were eight instances where queens sprayed hindgut fluid in a duel. They occurred in both a first duel and a second duel but never back to back and always between age-matched queens. The mean length of these duels was 73.3±20.2 minutes, approximately 20 minutes longer the mean duels lengths for all assays.

Not all assays commenced with immediate aggression between virgin queens. There were eight trials in which queens exhibited tolerance of their opponents. The combatants encountered each other without any attempts of grappling or stinging. My observations indicated that virgin queens may sometimes coexist without the onset of combat in this assay. Sometimes, one queen would crawl over the other without an onset of aggression. With one exception, this tolerant behavior was always documented in the second duel. As with spraying, the mean lengths of these duels (81.9±10.3 minutes) were substantially higher then mean lengths for all assays. Once again, only in two instances did the queen win both duels.

The most unusual phenomenon I observed was a trophallaxis-like exchange (the exchange of food between bees) between dueling queens - this behavior appeared in four assays for both duels (once in the first duel, three times in the second). One queen remained stationary as she presented her proboscis. The other queen inspected and subsequently paired her proboscis to her opponent. The behavior lasted from several seconds to nearly a minute (Figure 8). Subsequently, the queen who initiated contact won both duels in 3 of the 4 assays.

Only a small percentage of queens won both duels (Figure 2). In total, only 30% of the queens who won their first duel won their second duel. Twenty-six percent of the queens tested with the self-selection protocol won the second duel, with 24% winning in assays with a 15-minute interval and 25% winning in assays with a 50-minute interval. Half of the queens that were tested with the random-selection protocol were successful in the second duel. It should be noted however that there were only four assays that used the random selection protocol and they all had a 15-minute interval.

A logistic regression model was produced to determine how well duel lengths predict outcome (i.e. whether a focal queen won or lost). Less than 3% of the variance was explained by duel lengths. The model's predictive ability without the inclusion of duel lengths was 69.7 %. The Omnibus Tests of Model Coefficients produced a sig. value of .776. In addition, the Hosmer-Lemeshow Goodness of Fit Test provided a sig. value of .269, supporting that the model fits the data. With the inclusion of the predictor variables, the model predicted 69.7 % of the assays correctly, no change from the 69.7% initially predicted. Neither length of duel 1(Figure 6) nor length of duel 2 (Figure 7) are strong predictors for duel outcome. The Mann-Whitney Test determined that there is no significant difference in the lengths of the second duel for queens who only won their first duel and queen who won both , U=94.5000, z=-.804, p=.422, r=.14.

To explore the presence of winner effects, one-tailed binomial tests were performed with the alternate hypothesis that more than 67% of the queens under self-selection would win their second duel. The random selection assays were not statistically analyzed due to insufficient sample size. There was no evidence of a winner effect in the self-selection protocols. The tests indicated that a winner effect was not detected for self-selection, self-selection with a 15-minute interval, or self-selection with a 50-minute interval. To determine if prior experience in general had a significant impact on the outcome of the second duel, two-tailed binomial tests were performed for each self-selection protocol (Table 1). The tests revealed that experience in the first duel did significantly influence outcome. Instead of increasing winning probability however, prior experience decreased winning probability.

A post-hoc power analysis was utilized to determine if there were a sufficient number of queens to detect an actual effect in self-selection trials. The odd ratio indicated that the effect size is .191. The power analysis revealed that to replicate this effect at a power of .8 requires 171 queens. The post-hoc analysis can be problematic because it may erroneously imply that an effect was real but was undetected due to low sample size (Levine et al., 2001). Because my experiment yielded negative results, I also used a confidence interval to interpret the data because this measurement provides a more accurate assessment of small sample size than post-hoc analysis (Levine et al. 2001). For the 31 self-selection assays, the probability of a queen winning both duels was 0.258 (95% CI, 0.135-1.0). Because the confidence interval does not include the null value of 1, I cannot rule out the possibility that there is a difference between queens who won both duels and queens who only won their first duel. This suggests that the sample size was appropriate for measuring winner effect for the self-selection assays.

**DISCUSSION**

This study produced no evidence of a winner effect in honey bee queen duels. This was true both for queens with 15 and 50 minute intervals between duels and for duels conducted under the self-selection and random selection protocols, though sample sizes for the random selection protocol were inadequate to test for statistical significance. Despite having prior fighting experience and still displaying highly aggressive behaviors, focal queens were not better at fending off novice competitors.

The results refute a residency effect developing under dueling conditions and improving focal queens' winning probabilities, at least to the extent that the conditions of this behavioral assay reflect the more natural conditions of the beehive. While the duels occurred in neutral Petri dishes, it was possible that the interval period between the first and second duel could provide sufficient time for a residency effect to manifest for the focal queen. Instead, the extra time spent in the Petri dish did not promote greater success.

A tactic that virgin queens may employ during their duel is to release hindgut fluid (Tarpy et al., 2004). This "spraying" behavior only occurs during duels and has been hypothesized to be a defense mechanism, although its purpose remains undetermined (Tarpy & Fletcher, 2003). Other hypotheses have also been proposed for spraying. However, more observations are necessary to explore the adaptive nature of this behavior. Tarpy and Fletcher (2003) noted that the mean duel time for queens increased significantly when spraying events occurred. My results are consistent with their findings and providing further evidence that hindgut ejaculation is associated with extended duration. Tarpy and Fletcher (2003) suggested that spraying might increase the survivorship of the queen who releases the compound. But

because queens move rapidly before they spray and because there are no prior behavioral cues, it was impossible for me to determine which queen(s) sprayed. Moreover, only in two instances did the queen win both duels.

Trophallaxis normally occurs among workers or between workers and the queen (who does not provide food to worker bees) and is considered one of the most cooperative interactions between bees. A bee thrusts the tip of her tongue towards a nearby bee who will then open her mandibles and offer food; typically in the form of fluid from the honey stomach (Winston, 1987). Further research is required to understand why a subset of competing queens engaged in this behavior.

In regards to the queens who were placed in the fighting arena but did not attempt to grapple or sting each other, genetic variation may be a significant contributing factor. The virgin queens were the offspring of queens who were naturally mated, presumably with many different drones, and perhaps they carried alleles associated with extreme docility. It is possible that these queens were derived from drones with the most docile genotypes and random pairing created these unique combinations.

Experience effects tested under both self and random selection can be transient in insects (e.g. Benelli et al., 2015, Rillich et al., 2011) and last for only several minutes. The disruptive nature of virgin queen duels is physically taxing and might necessitate the winner time to recuperate for her next encounter. I assumed that a 15-minute interval between duels was an optimal accommodation that provides the focal queen time to recuperate from her previous encounter while observing for a winner effect with a potentially limited timespan. However, winner effect can also last for extended period and more time to recuperate may lead to further manifestation of experience effects. Thus, a 50-minute interval tested the upper limits of winner

effects. These interval lengths were also used for *Eupelmus vuilleti,* the first of two hymenopterans to be tested for winner/loser effects (Goubault & Decuignie`re, 2012). No differences were observed in my study for these two different interval periods.

Contest experience is just one of many factors contributing to contestant behaviors and contest outcome (Hsu et al., 2006). One factor of consideration is the fate of the losers in the competition. Unlike other species that have been observed for winner and loser effects, the honey bee queen is unique in that outcome is decided by death of the opponent and not by which individual is the first to retreat or surrender.

According to the Sequential Assessment Model, the value of experience effects tends to decrease as agonistic behaviors become more physical and the contestants can directly compare actual RHP (Hsu et al., 2006). Because queen duels are quickly physical without an escalation period, it is possible that the contribution of experience effects disappears almost immediately. The physically taxing and lethal nature of these duels compared to other species leads to higher instances of winners sustaining injuries that would impair their physical abilities; potentially to the extent that experience cannot compensate. This would explain why queens who fought for more than 1 hour in the first duel typically lasted less than 15 minutes in their second duel (Note: all of these queens had a 15 minute interval). The combination of exhaustion and injury may also explain why prior fighting experience seems to lower winning success for virgin queens. Therefore, it may be beneficial for virgin queens to engage in the least number of duels to increase their survivorship and probability of colony inheritance.

Findings that suggest that queens that emerge earlier (and thus have more interactions with opponents) are more likely to be winners are only relevant in colonies without after-swarms (Schneider & DeGrandi-Hoffman, 2007). Queens that inherit their nests that produced after-

swarms were the last to emerge. Therefore it seems that the contribution of prior experience to a successful outcome is only relevant when virgin queens do not depart in subsequent swarms.

Completely eliminating defeated opponents in this way makes it impossible for there to be a loser effect, which tends to be strongly associated with a winner effect. One exception is the work of Goubault and Decuignie`re (2012) who postulated that a change in subjective resource value (SRV) led to the solitary winner effect in the parasitoid wasp *Eupelmus vuilleti* in the absence of a loser effect. In this species, finding a host leads to physiological changes, such as increase in egg load. A wasp who is a winner is more likely to oviposit and more willing to defend the resource: producing a winner effect. However, a loser would not change its value of the host and thus a loser effect does not occur. There is no evidence that virgin queen honey bees modify their perception of the hive's value and thus experience effects via queen competition would not manifest in this manner.

Queens that were successful in both duels likely possess higher resource holding potential than the others; that is their success may be attributable to RHP (which includes body size), without any effect of experience. It is possible that winner effects may not manifest in the bees because there may be no opportunities for selection to act on mechanisms that drive it. Once again, further research is needed to address this.

Dietmann et.al (2008) demonstrated that honey bee queens employ self-assessment during duels. They use prior information of their status to estimate fighting ability and modify behavior for subsequent opponents. In addition, the costly nature of mutual assessment lends to self-assessment to being a more beneficial tactic. Despite this, there was no evidence from the majority of focal queens that they modified their fighting behaviors in the second duel. Only in a small percentage of queens were methods employed that are possibly associated with increased

survivorship (spraying, trophallaxis, retreating, etc.). On the other hand, prior winning experience may have raised their estimated fighting ability to a level that eclipsed their recognition of current physical status, thus decreasing their attention to prior injuries. This is consistent with the idea that winner effects are diminished due to the cost of overestimating one's fighting ability (Goubalt and Decuigniere, 2012). Therefore, there might be a modification of the queens' RHP perception but not strong enough to increase fighting success.

An alternative means to explore for the presence of winner effects (and possibly also loser effects) in honey bees would be to blunt the sting of the queen bees before dueling so they cannot inflict serious damage to their opponents. However, this leads to behavioral changes that are atypical to what is observed under natural and other experimental conditions; such as older queens not attacking young combatants and early cessation of stinging/grapping (Palelog, 2011).

A problem in this study was the low number of one-day-old queens produced for random selection. The intent of this project was to produce equal number of assays using self-selection and random-selection. Due to time restraints and limited queen rearing abilities however I produced a small sample size of one-day-old queens and therefore conducted only 4 random selection assays. The random selection technique is advantageous in that it produces winners with wide ranges of resource holding potential and thus is somewhat more accurate in detecting winner effect contributions. Self-selection however is advantageous in that it can be used when true predictors of fighting ability are unknown or not fully assessed and this technique is not hindered by the stringency required for random-selection (i.e. ensuring that there is considerable discrepancy in expected fighting abilities between opponents). Additionally, studies that explored winner and loser effects in insect species found that differences in intrinsic fighting

ability were not major factors for confounding prior fighting experience (e.g. Hsu et al., 2006, Wilgenburg et al., 2010, Adamo & Hoy, 1995).

Another limitation of this study is that I did not graft one-day-old-queens in queen builders distinct from the ones that produced older queens due to concerns of behavioral artifacts. It is possible that virgin queens respond differently to virgin queens produced from different colonies and I would have no means to discern these interactions.

If future research provides evidence of winner effects in queen honey bees, this would open the opportunities to explore other aspects of the phenomenon. For some species, the mere exposure of fighting was enough to elicit a winner effect. In other words, subjects experienced increased fighting success in subsequent duels despite the absence of a clearly identifiable winner or loser in their first contest (Rillich & Stevenson, 2011). This could be tested in virgin queens by placing them in arenas that allow for limited grappling but prevents the queens from delivering stings. After a given time period, both queens would be removed from the dish and placed in separate unmodified dishes with a naïve opponent to fight. If repeated experiments show that both queens tend to defeat their separate opponents, this would suggest that contest experience is sufficient to produce an advantage in future duels.

Of great interest would be to test the extent of winner effects. Using the random selection protocol, queens grafted from low-quality larvae (e.g. two-day-old larvae) could be selected as winners, aged, and pitted against newly emerged queens. After these duels, the low-quality queens could be paired against age-matched high quality queens (e.g. queens grafted from one-day-old larvae) who have no contest experience. If the low-quality queens consistently defeat the high quality queens, this would provide evidence that the winner effect can confer an advantage

to queens with reduced physical fitness and that these queen have the capacity to inherit their colony.

Contributions of a social queen to her progeny extend beyond genetic material. Wright et al. (2017) explored the relationship between queen paper wasps (*Polistes metricus*) and their offspring in terms of behavioral patterns. Their observations revealed that queens that were less likely to leave their nest after multiple encounters with an antagonistic stimulus produced more workers than "shyer" queens. Shyer queens in turn were more likely to produce workers that attacked more frequently when disturbed. This suggests that "personality" aspects of paper wasp queens can permeate in the colony via her reproduction rate and the collective behavior of her offspring. Observing and studying honey bee queen duels may provide researchers and beekeepers an ecological snapshot of the potential size and conditions of future bee colonies.

This project has provided a broader perspective of behaviors demonstrated by conspecifics whose outcome can have permanent ecological and genetics implications for an environmentally and economically critical species. It has also provided evidence that honey bee queen duels represents a complex phenomenon in which differences in physical fitness may contribute more to outcome than differences in contest experience. Because western honey bees provide immeasurable services to crop production and pollination, it is important to have a better understanding of all aspects of queen biology. This project has further increased our knowledge of both the direct and subtle behavioral displays produced between potential reproductive leaders of an obligate eusocial system in conflict.

**Table 1:** Statistical analysis (binomial test) for differences in the percentage of queens who won their second duel.

**Self-Selection (Both 15 and 50-Minute Intervals)**

| | | Category | N | Observed Prop. | Test Prop. | Asymp. Sig. (2-tailed) |
|---|---|---|---|---|---|---|
| Outcome | Group 1 | Win | 8 | .26 | .66 | **.000**[a] |
| | Group 2 | Lose | 23 | .74 | | |
| | Total | | 31 | 1.00 | | |

a. Based on Z Approximation.

**Self-Selection with 15-Minute Interval**

| | | Category | N | Observed Prop. | Test Prop. | Asymp. Sig. (2-tailed) |
|---|---|---|---|---|---|---|
| Outcome | Group 1 | Win | 4 | .24 | .66 | **.000** |
| | Group 2 | Lose | 13 | .76 | | |
| | Total | | 17 | 1.00 | | |

**Self-Selection with 50-Minute Interval**

| | | Category | N | Observed Prop. | Test Prop. | Asymp. Sig. (2-tailed) |
|---|---|---|---|---|---|---|
| Outcome | Group 1 | Win | 4 | .29 | .66 | **.010** |
| | Group 2 | Lose | 10 | .71 | | |
| | Total | | 14 | 1.00 | | |

| | |
|---|---|
| **Win** | Queen won 1st and 2nd duels |
| **Lose** | Queen won 1st duel, lost 2nd duel |
| **N** | Number of queens in each category |
| **Observed Prop.** | Actual proportion of queens for each category |
| **Test Prop**. | Expected proportion of queens who won both duels |

**Figure 1:** Experimental design for queen duel study. Two queen duels (right) are occurring, while the naïve conspecifics who will fight the winners are placed in separate dishes (left) facing the wall. Vial tubes were used for transporting combatants and randomly assigning queens to their fighting groups.
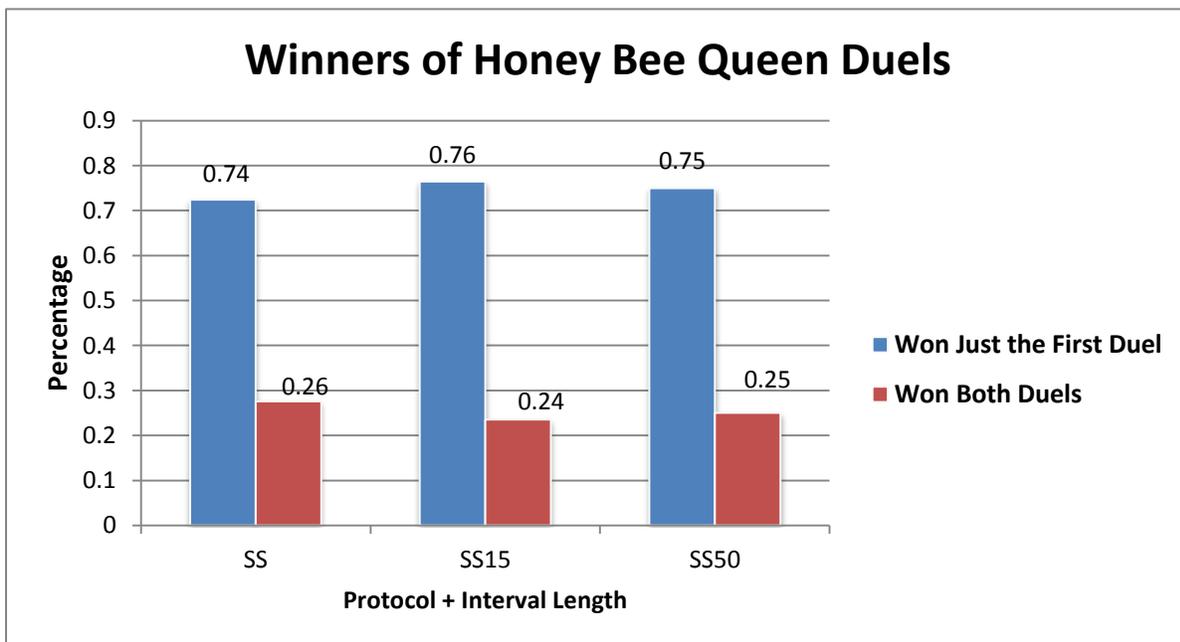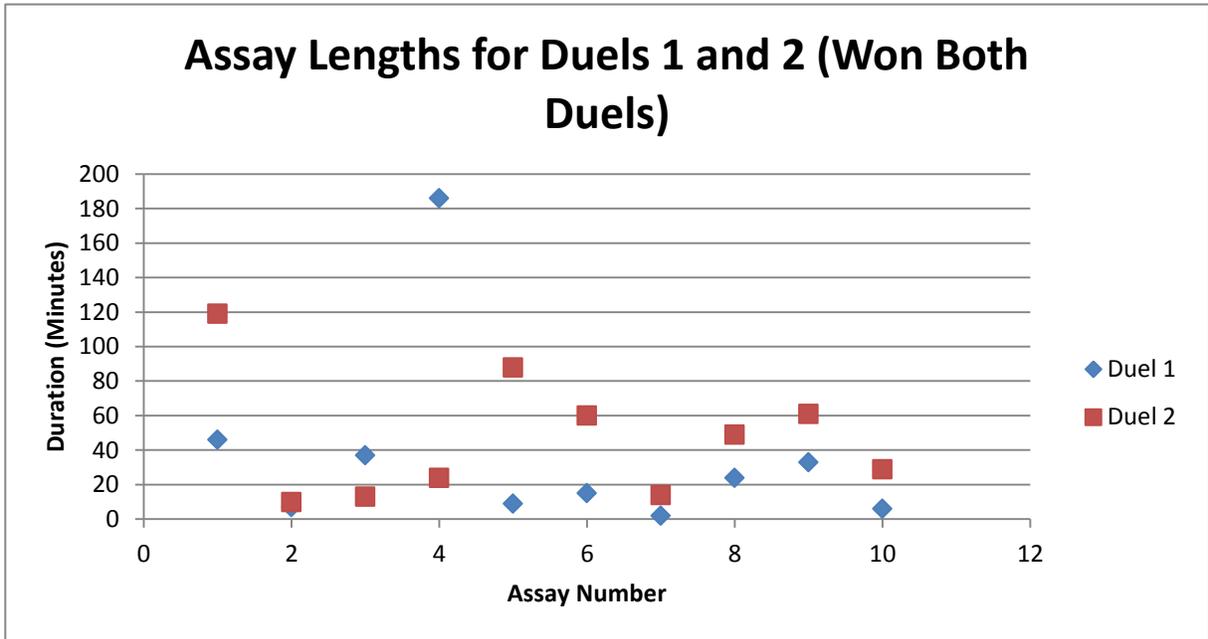


**Figure 2:** Percentage of queens who won both duels as a function of assay protocol and inter-duel interval.
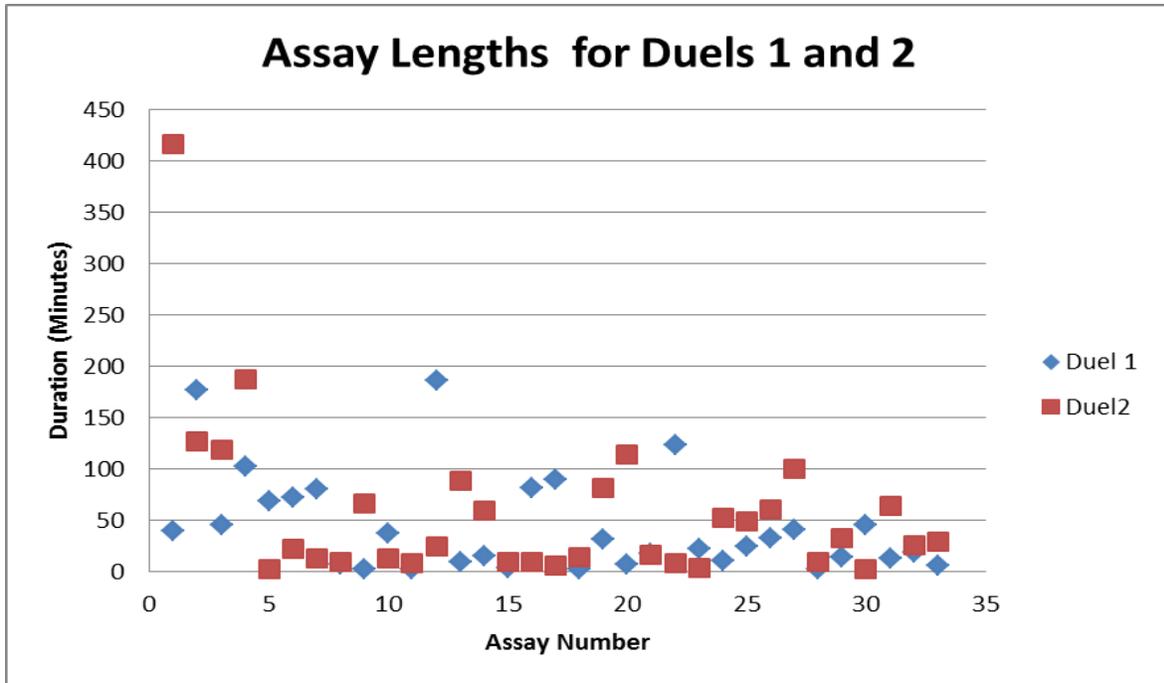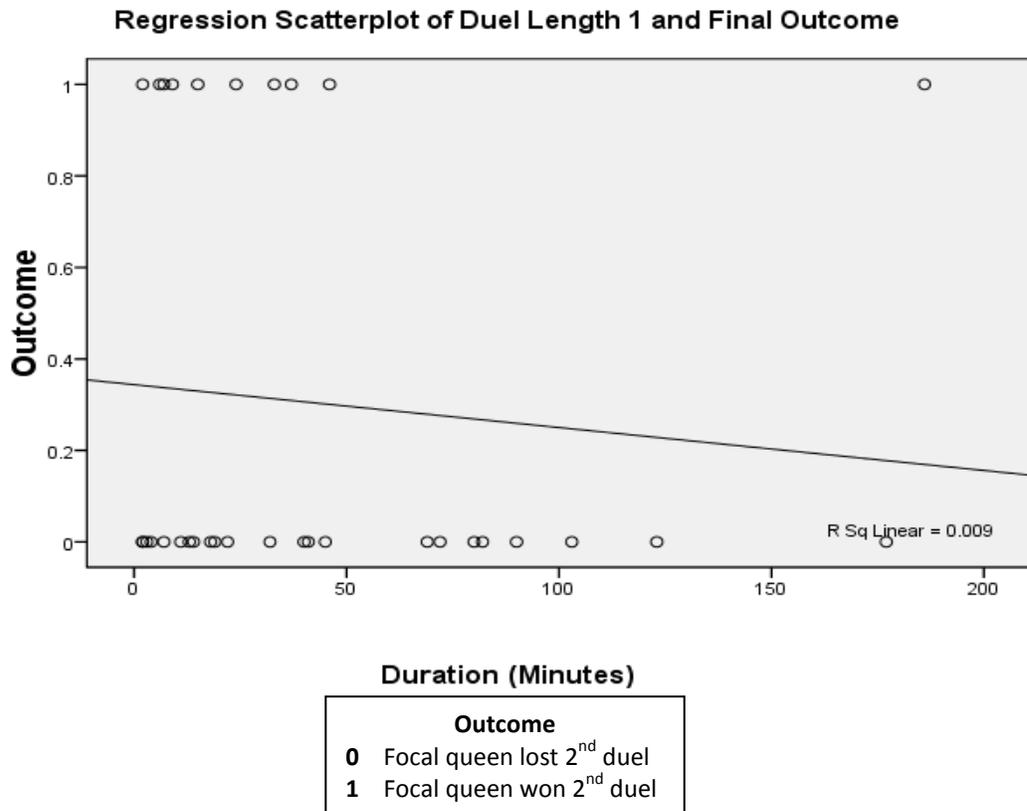
**Figure 3:** Lengths of duel 1 and duel 2 for all assays in this study.
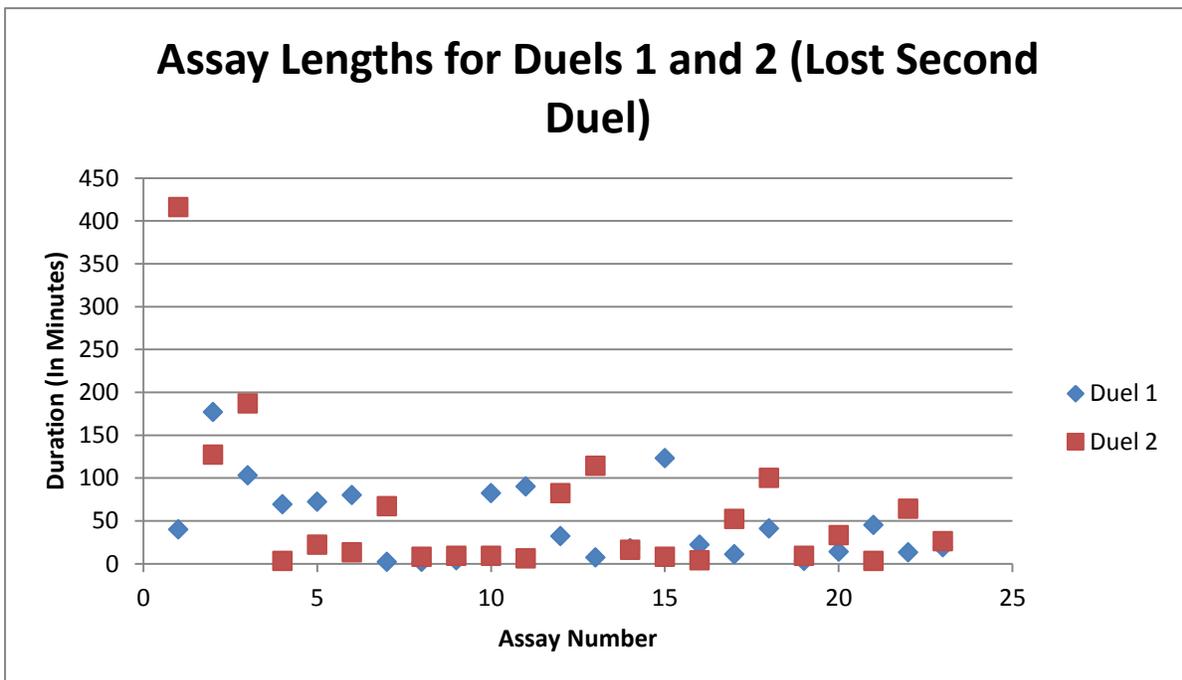


**Figure 4:** Lengths of duel 1 and duel 2 for assays in which the focal queen lost the second duel.

**Figure 5:** Lengths of duel 1 and duel 2 for assays in which the focal queen won her first and second duel.



**Figure 6:** Scatterplot displaying the relationship between the duration of time the focal queen spent in her first duel and her outcome in the second duel (win or lose).
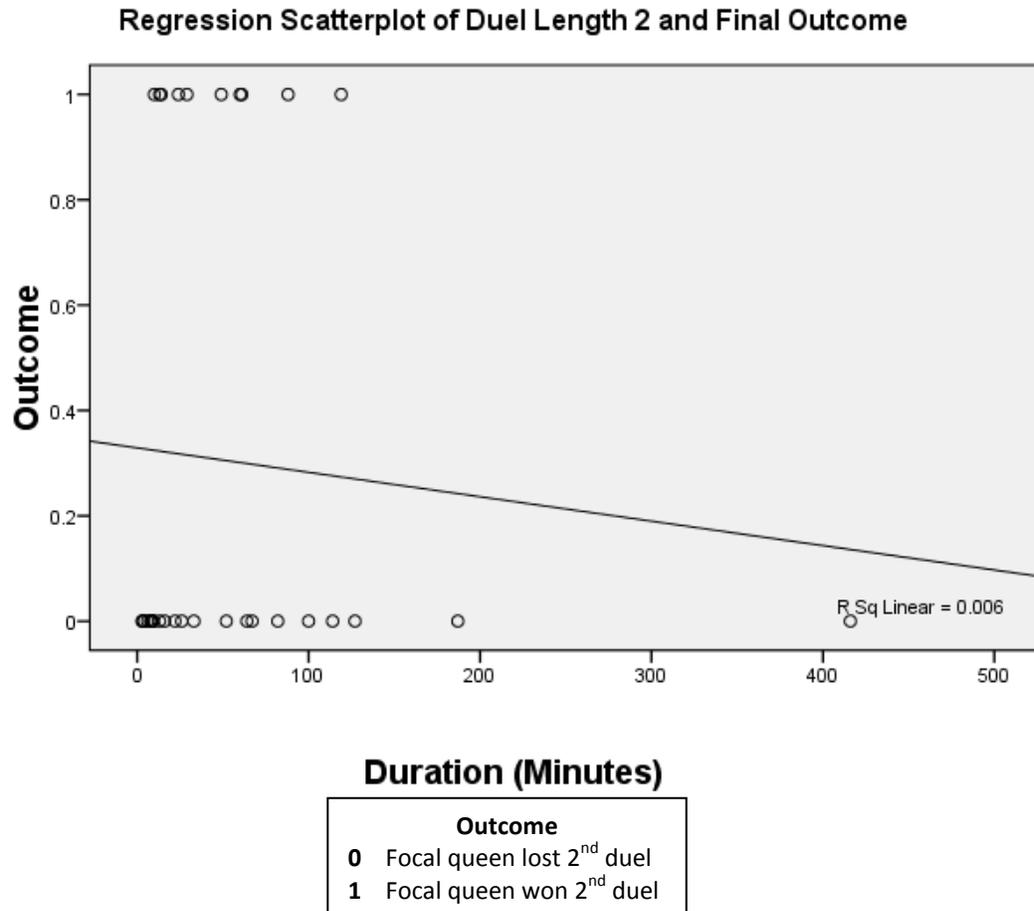
**Figure 7:** Scatterplot displaying the relationship between the duration of time the focal queen spent in her second duel and her outcome in the second duel (win or lose).



**Figure 8:** Queen competitors displaying proboscis presentation behavior.

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