The Innate Immune Response of Eusocial Hymenopterans to Viral Pathogen Challenge

V. Renee Holmes¹, and J. Spencer Johnston

Department of Entomology, Texas A&M University, College Station, TX 77843, USA and ¹Corresponding author, e-mail: vrh0933406@tamu.edu

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Abstract

In recent years, insect immunology has expanded rapidly in research interest, and available literature has expanded in kind. Insects combat pathogens through a range of behavioral and physiological immune defenses. The need for robust immunity is especially important to eusocial insects; nestmate proximity increases exposure to and transmission of pathogens. Further, eusociality involves cohabitation of thousands of individuals with characteristically reduced genetic variability, in turn increasing susceptibility to epidemic disease outbreaks. To combat this, they have developed diverse responses to pathogens, including individual innate immune defenses, social immunity, and secretion of potent glandular chemicals. The range of immune responses is as diverse as the pathogens presenting the threat, and of these, viruses may present the greatest challenge. Social immunity employed by Hymenoptera and has been reviewed whereas a review has not been developed to our knowledge addressing innate immunity of eusocial Hymenopterans to viral pathogenic invaders. We argue that such a review is important to advancement of understanding of Hymenopteran biology and is critical to applied interests. We argue further that the implications of eusocial Hymenopteran innate immunity are far-reaching; their success is a source of both substantial economic loss in the case of invasive ants and significant economic gain in the case of the honey bee Apis mellifera.

Key words: immunity, eusocial Hymenoptera, virus

Viral pathogens are among the most important and least thoroughly understood of the immune challenges faced by eusocial insects (Lester et al. 2019). Viruses in the family Soliviviridae are natural enemies of the major eusocial insect pests the red imported fire ant Solenopsis invicta (Valles et al. 2004, Valles and Rivers 2019) and the tawny crazy ant Nylandria fulva (Hymenoptera: Formicidae) (Valles et al. 2012). Introduction of these pathogens to invasive ranges is an area of biological control interest. At the same time, the honey bee Apis mellifera (Hymenoptera: Apidae) is plagued by colony collapse disorder associated with viral infection (Chejanovsky et al. 2014), and colony success is dependent on ability to respond to challenge by these pathogens. Invasive ant species are responsible for billions of U.S. dollars in losses annually to a range of economic interests (Lard et al. 2006, Adams 2019). Conversely, the honey bee is one of the most important insects in terms of economic contribution, and maintaining their health in response to viral pathogens that plague them is important to innumerable interests (Genersch 2010). This review builds on the recent and growing body of work detailing the immune responses of eusocial insects, with primary interest focused on the innate immune system. We start by briefly describing social immunity at the colony level, paying particular attention to colony scale defenses. We then narrow the scope to the individual level, focusing on the innate immune response. We present a compilation of the ever-increasing body of literature and research on eusocial innate immune response to viral pathogens, and offer some new questions to pursue in future work within this exciting field.

Eusociality in insects has evolved independently multiple times, most notably in termites and Hymenoptera (Nowak et al. 2010, Quiñones and Pen 2017). Despite the benefits of eusociality, the environment created by communal living also creates a conducive scenario for parasites and pathogens; constant availability of food sources, presence of vulnerable immatures, and hospitable ambient temperatures provide an exceptional environment for high pathogen and parasite loads (Feldhaar and Gross 2008). Increased contact between nestmates can lead to increased opportunity for pathogen transmission, and increased relatedness classic to the eusocial
structure results in similar susceptibility to pathogens (Baracchi et al. 2012b). This combination of characters is highly conducive to epidemic disease outbreaks (Quevillon 2018, Pinilla-Gallego et al. 2020). For these reasons, the emergence of the eusocial colony structure fostered the need for a strong response to a range of invaders, both pathogenic and parasitic. Eusocial insects meet this challenge by many avenues, from broad-scale social immune response to individual, chemically mediated innate immune response.

Because invertebrates, including eusocial insects, do not have a true antibody-mediated immune system, their success is greatly influenced by their ability to function as a unit in colony-level immune response (social immunity), and at the individual level via innate immune responses (Erler et al. 2011, Myllymäki and Rämert 2014, Palmer and Jiggins 2015). Throughout history, an evolutionary “arms race” has unfolded between insect hosts and the pathogens infecting them (Erler et al. 2011). Insect response to pathogens on an immunological level is an area of interest that has been well studied in mosquitos and the Dipteran model organism Drosophila (Diptera: Drosophilidae) (Palmer and Jiggins 2015). Recent interest focusing on immunity in social Hymenoptera reflects a growing awareness of their importance to human interests.

Superorganism-Level Social Immunity

The term “social immunity” refers to the behavioral adaptations and modifications that contribute to both the individual and colony level defenses against infection. Social immunity is dependent on the cooperation of the colony to avoid, mitigate and clear parasitic infections (Cremer et al. 2007, Cremer and Sixt 2009, Erler et al. 2011, Cremer et al. 2018). Social insects display an impressive repertoire of these behavioral, colony-wide immune defenses. Mating strategies may be among the first defenses against pathogen threat; social insect female reproducitives are largely monogamous and mate in a single event, thereby reducing transmission of pathogens and parasites to the primary reproducitives and diminishing potential for vertical pathogen transmission (Cremer et al. 2007). A. mellifera immune response to invaders starts outside of the colony with specialized gatekeeper honey bees stationed at the colony entrance to deny infected or parasitized individuals access to the nest (Waddington and Rothenbuhler 1976, Drum and Rothenbuhler 1985, Cremer et al. 2007). When the first line of checkpoint defenses is inadequate to prevent enemy invasion, a host of other social immune responses including allogrooming for parasite removal, social fever to heat-kill bacteria, and prophylactic uptake of resins that support and increase chemical resistance to infection are deployed (Cremer et al. 2007). Social fevers in ants (Cremer 2019) and honey bees (Goblirsch et al. 2020) can effectively cook microorganism and parasitic invaders, either neutralizing or reducing the threat posed by such invaders (Sugahara and Sakamoto 2009, Goblirsch et al. 2020).

Antimicrobial peptides also contribute broadly to both social immunity and individual immune response; honey bee venom has antimicrobial properties when applied to the cuticle and deposited in the comb (Baracchi et al. 2011, Baracchi et al. 2012a, Moreau 2013, Simone-Finstrom 2017). A similar phenomenon is observed in select ant species, in which cuticular application of venom appears to have sanitary implications (Tragust et al. 2013, Simone-Finstrom 2017). Hygienic behaviors, including recognition and removal of diseased adults (Baracchi et al. 2012b) and the hygienic removal of ectoparasites and pathogens serves as a preventative action in a broad range of foraging eusocial Hymenoptera including the western honey bee A. mellifera (Bozic and Valentinic 1995), Asian honey bee Apis cerana (Hymenoptera: Apidae) (Rath 1999), and leaf-cutting ants Acromyrmex subteraneus (Hymenoptera: Formicidae) and Acromyrmex octospinosus (Hymenoptera: Formicidae) (Richard et al. 2009).

While superorganism level social immunity is highly beneficial and critical in initial response to pathogen invasion, it is often insufficient to overcome viral pathogenicity in the absence of innate immune response (Brutsch et al. 2015). The lack of major histocompatibility complexes, T-receptors, or immunoglobulins characteristic of vertebrate immune systems leads to a heavy reliance on individual-level mechanical barriers to pathogens and innate (chemically mediated) immune responses. Among the known pathways implicated in eusocial insect innate immunity to viral pathogens are the immune deficiency (Imd), Toll, RNAi, and Janus kinase (JAK/STAT) signaling pathways, as well as the secretion of antimicrobial peptides (AMPs) (Barrieau et al. 2015, Brutsch et al. 2015, Brutsch and Flenniken 2015).

Immune Deficiency (Imd) Pathway

In eusocial Hymenoptera, the involvement of the immune deficiency or Imd pathway in defense against viruses is somewhat cryptic, and the overwhelming majority of research on insect Imd is limited to Drosophila. The Imd pathway plays a complex role in insect life history, acting as both an immune response pathway and an important regulator in development (Erler et al. 2011). In the immune system of Drosophila, Imd is responsible for the regulation of the NF-κB protein Relish (Dushay et al. 1996, Myllymäki and Rämert 2014), and expression of the majority of Drosophila antimicrobial peptides (AMPs) (Myllymäki et al. 2014). In Drosophila, activation of the Imd pathway commences with the recognition of microbial agents, facilitated by detection of pathogen-associated molecular patterns (PAMPs) that are unique to the pathogen. Imd activity is most commonly associated with bacterial pathogens, specifically Gram-negative and certain Gram-positive bacteria, as PGRP-LC (transmembrane receptor) preferentially binds to a mesodiaminopimelic-acid unique to bacteria (Kaneko and Silverman 2005, Klein and Silverman 2014, 2019). It acts as the principal receptor in initiation of the Imd pathway in systemic infection, or in localized midgut response in the anterior section of the midgut in localized infection (Choe et al. 2002, Gottar et al. 2002, Zaidman-Rémy et al. 2006, Myllymäki et al. 2014). Among the common recognition factors is peptidoglycan, a structural component of many bacterial pathogens. Curiously, ectopic expression of PGRP in Drosophila fat body can activate expression of antimicrobial peptides in the absence of infection (Myllymäki et al. 2014). Involvement of the Imd pathway varies depending on the infective agent species; Imd plays a greater role in Drosophila defense against Sindbis virus and Cricket Paralysis Virus than does Toll, whereas Imd does not appear to be heavily involved in clearance of Drosophila C Virus (Brutsch et al. 2015).

Recent work is supportive of the implication of Imd in response to viruses. Molecular work investigating viral immune response in the Argentine ant Linepithema humile (Hymenoptera: Formicidae) revealed a gradient of response to different types of pathogens. L. humile transcriptomic analysis of immune response to bacterial Pseudomonas spp. (Pseudomonadales: Pseudomonadaceae) infection and L. humile virus 1, whereas the honey bee pathogen Black Queen Cell Virus did not elicit a major alteration in immune pathway expression (Lester et al. 2019). Relish, a transcription factor in the Imd pathway, has been cloned and sequenced from both
Toll Pathway

As is the case with many insect immune pathways, the majority of work done on insect Toll has focused on *Drosophila*. Toll is a highly conserved pathway across genera and is integral to both the immune response and developmental processes of vertebrates and insects (Evans et al. 2006). Toll is most commonly implicated in fungal and Gram-positive immunity in insects, but evidence shows that viral immune response can be incurred by Toll signaling as well (Doublet et al. 2017, Rosales and Vonnie 2017, Lester et al. 2019, Baty et al. 2020). In *Drosophila*, Toll is initiated by the cleavage of the cytokine-like transcription factor Spatzle and the binding of the C-terminal fragment of the leucine-rich repeat (LRR) of Toll (Weber et al. 2007, Lindsay and Wasserman 2014). When Spatzle binds to the Toll LRR, Toll dimerizes and becomes active. Following activation, the Toll/interleukin-1 receptor homology (TIR) domains dimerize, which promotes binding of adapter protein MyD88 via its TIR domain. MyD88 then binds adapter protein Tub and recruits the protein kinase Pelle which binds to death domains. Pelle recruitment leads to autophosphorylation of Pelle, which induces degradation of the inhibitor Cactus. Degradation of Cactus induces release of transcription factors either Dif or Dorsal, which are then translocated to the nucleus (Lindsay and Wasserman 2014).

Toll is also implicated in the immune response of eusocial Hymenoptera. Notably, there are two plausible Spatzle orthologs evident in the honey bee genome (GB13503, GB15688) (Evans et al. 2006). Sequencing of the honey bee genome also revealed two homologs of the *Drosophila* transcription factor Dorsal, and the intracellular components Cactin, Pellino, TNF receptor-associated factor-2, and Tollip. All of these components are believed to play major roles in the Toll pathway, and all appear to be present in both *Drosophila* and honey bee species (Evans et al. 2006, Doublet et al. 2017). Transcriptional studies have implicated Toll in a complex role in viral response in the honey bee; young bees experimentally infected with Israeli Acute Paralysis virus (IAPV) exhibited increased expression of Toll genes whereas more mature, naturally infected bees did not show transcriptional implication of Toll (Brutscher et al. 2015, Galbraith et al. 2015). While ant viral immune response remains poorly understood, recent work in the Argentine ant *L. humile* also supported the belief that a core set of immune genes are involved in ant immune response to pathogen challenge (Lester et al. 2019). Toll pathway NF-κB homolog dorsal-1A was transcriptionally induced in worker caste honey bees when parasitized by the mite *Varroa*, strongly suggesting that Toll is implicated in response to Deformed Wing Virus (DWV) infection, as *Varroa* is responsible for DWV transmission to honey bees (Doublet et al. 2017).

Other components of the Toll pathway have been identified in eusocial Hymenoptera as well. The transcription factor Relish has been cloned and sequenced in ant species *Nothonyrmecia macrops* as well as several *Myrmecia* species, (Schlüns and Crozier 2009, Johansson et al. 2013, Lindsay and Wasserman 2014). Similarly, the Toll signaling pathway components Toll, Pelle, and Dorsal have been cloned and sequenced in *Formica aquilonia* (Hymenoptera: Formicidae) (Lindsay and Wasserman 2014).

The Toll signaling pathway has also been implicated in the immune response of globally invasive ants to challenge of viral pathogens. Lester et al. 2019 used RNA sequencing analysis to identify viruses infecting invasive Hymenopterans. The most strongly associated immune genes involved in positive-sense RNA viral infection (Deformed Wing Virus and *Linepithema humile* virus-1) are peptide recognition proteins assigned to the Toll and Imd pathways in the invasive Argentine ant (Lester et al. 2019). This indicates strong promise for similar involvement of the Toll pathway in the red imported fire ant response to *Solenopsis invicta* viruses, as these viruses are also positive-sense, single-stranded RNA viruses (Valles et al. 2004).

Antimicrobial Peptides

Antimicrobial peptides (AMPs) are diverse, highly conserved, and crucial effectors present in a variety of vertebrate and invertebrate immune systems. AMPs are implicated in immune response against a number of pathogen groups, and act in response to the microbial membranes of the pathogens. They generally consist of 15–20 amino acids and are classified based on their amino acid structure and composition (Wu et al. 2018). While AMPs are primarily effectors against bacterial pathogens, some are effective in response to viral challenge (Evans and Lopez 2004, Yi et al. 2014, Wu et al. 2018). Among these are cecropins, which were originally identified and isolated from the hemolymph of the cecropia moth *Hyalophora cecropia* (Lepidoptera: Saturniidae) from which the name is derived (Hultmark et al. 1982). While cecropins are largely implicated in defense against Gram-positive and Gram-negative bacteria, cecropin P1 inhibits viral particle release and attenuates virally induced apoptosis (Schlungs and Crozier 2009, Guo et al. 2014, Wu et al. 2018), thereby reducing viral dissemination. The mechanism of action involves disruption of the viral envelope and shows significant inhibitory action against viral particle release (Schlungs and Crozier 2009, Guo et al. 2014, Wu et al. 2018).

A number of AMPs have been identified in ants; defenses were recognized via cloning and sequencing in 25 formicine ant species and 2 *Myrmica* species (Schlungs and Crozier 2009). Among the identified AMP-associated genes is one that codes for a serine protease inhibitor that could be implicated in immune signaling (Schlungs and Crozier 2009). Two AMPs that are similar to hymenoptaecin and abecacin from the honey bee A. *mellifera* have been identified in the red imported fire ant *Solenopsis invicta* (Tian et al. 2004). These two AMPs are expressed more strongly in newly dealated *S. invicta* queens than in unmated queens, suggesting an immune challenge associated with mating (Evans et al. 2006). AMPs have also been identified in the venom of some ants (Kuhn-Nentwig 2003). Among these are pilosulins, which show similarity to melittin from A. *mellifera*. Melittin is antimicrobial and hemolytic (Schlungs and Crozier 2009).
**RNAi**

RNAi, or RNA interference/silencing, is a highly conserved regulatory and defensive pathway by which *Drosophila* and other insects combat viral infection. The RNAi pathway proceeds by two main steps, initiation and execution (Van Rij et al. 2006, Zambon et al. 2006). In initiation, double-stranded RNAs (dsRNA) are processed by endoribonucleases Dicer1 or Dicer2 into smaller dsRNA segments with 3’ overhanging ends. The processed, smaller RNAs are used for initiation of RNAi execution steps (Zambon et al. 2006, Brutscher and Flenniken 2015, Brutscher et al. 2015). Following initiation, small RNA sections are incorporated into the RISC, or RNA-induced silencing complex. This involves binding of the template strand to an Argonaut protein. The anti-sense strand of the small dsRNAs are then incorporated into the RISC by protein t2d2 (Schwarz et al. 2004). The singular strand of the dsRNA incorporated into the RISC by Argonaut protein binding remains and acts as a guide to locate complementary mRNA. This is called the guide strand. Binding to mRNAs within the cell is precise due to guidance by base pairing between the strand bound to RISC and the target. Following binding, Argonaut initiates and catalyzes cleavage of the targeted mRNA, resulting in degradation. The process is similar when microRNAs are used as the guide strand in Argonaut, but miRNA guiding can result in imprecise binding and degradation of larger mRNAs (Schwarz et al. 2004). While vital to regulation of a number of endogenous mRNAs, RNAi binding and degradation is also involved in the control of viruses and is highly conserved in a variety of organisms including plants, invertebrates, and some bacteria (Robbins et al. 2009).

The siRNA pathway has been implicated in defense against viral pathogens in *Drosophila* and other insects. Flockhouse virus infection was shown to be minimally virulent against *Drosophila* in wild-type flies (50% survival 15 days postinfection), whereas Dicer-2 mutant flies showed 60% mortality 6 dpi and 95% at 15 dpi (Wang et al. 2006). The siRNA pathway is highly conserved and is also implicated in social Hymenopteran viral immune response. The majority of viral pathogens infecting the social Hymenopteran honey bee *A. mellifera* are RNA viruses in families *Dicistroviridae* and *Iflaviridae* (Niu et al. 2014). Deep sequencing performed on colony-collapse disorder suffering colonies identified abundant 21-22 nucleotide siRNAs with perfect sequence matching to Israeli Acute Paralysis Virus (IAPV), Kashmir bee virus, Deformed Wing Virus (DWV) (Hunter et al. 2010), and *A. mellifera* rabdoviruses-1 and -2 (McMenamin and Flenniken 2018). Reduction of viral titer of IAPV and DWV were demonstrated, supporting the assertion that the siRNA pathway is implicated in viral immune response as in *Drosophila* (Hunter et al. 2010). Similar results were observed when Israeli and United States honey bee colonies were experimentally challenged with the eight different viral species (Chejanovsky et al. 2014). Similarly, Florida and Pennsylvania honey bee colonies elicited significant protection from the negative effects of IAPV from homologous dsRNA exposure (Hunter et al. 2010). Increased expression of *dicer* and *argonaute-2* occurred with exposure to both an experimental model virus (Brutscher et al. 2017) and to the pathogen Israeli Acute Paralysis virus (IAPV) in the honey bee (Galbraith et al. 2015). *A. cerana* larvae treated with dsRNA specific to Chinese Sacbrood virus also resulted in reduction of viral titer of the virus present within sampled colonies (Brutscher et al. 2015, Brutscher and Flenniken 2015). These results are likely relevant to interests in invasive ant response to viral pathogens, as many pathogens infecting invasive ants are, or were previously, classified as members of the viral family *Dicistroviridae* (Lester et al. 2019, Baty et al. 2020).

**JAK/STAT**

In *Drosophila*, JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway ligands include three cytokine-like proteins named unpaired (upd), unpaired2 (upd2), and unpaired3 (upd3). All are induced by tissue wounding. Upd is induced by bacterial immune response, but upd2 and upd3 are induced as a response to viral pathogen detection. In response to pathogenic invasion, each of the upd molecules specifically binds to a unique receptor called Domeless. In *Drosophila*, there is one JAK (Avadhanula et al. 2009) and one STAT transcription factor, hopscotch and Stat92E, respectively. In response to the binding of a cytokine to a receptor, the receptor dimerizes and activates the JAK. The activated JAKs then phosphorylate one another as well as specific tyrosine residues on the receptor, which then act as docking sites for the Src homology 2 domains of the STAT molecule. STATs are also phosphorylated by the JAKs, allowing them to dimerize and be translocated to the nucleus to act as promoters of their target genes (Myllymäki and Rämö 2014).

The JAK/STAT pathway in insects has been best studied in *Drosophila*, which are plagued by a number of viral natural enemies. As is the case in the insect response to other pathogens, the *Drosophila* immune response to viral pathogens involves cross-talk between multiple systems, including the Toll and JAK/STAT. The immune response genes induced by fungal and bacterial pathogen invaders are distinct from those induced by viruses, suggesting a specific response system tailored for the invaders they target (Myllymäki and Rämö 2014). It has also been noted that many genes are activated by the JAK/STAT pathway in response to *Drosophila* C virus, and viral loads and mortality increase with deficiencies in this pathway (Myllymäki and Rämö 2014).

It has been demonstrated that *Drosophila* expression of cytokines Upd2 and Upd3 are strongly induced following infection with Cricket Paralysis Virus (family *Dicistroviridae*) (Lamiable and Imler 2014). This is relevant to immune studies in invasive ants and honey bees, as many of the pathogens infecting these eusocial Hymenoptera are members of *Dicistroviridae*. The fire ant pathogens *Solenopsis invicta* viruses were originally placed within the same viral family...
(Valles et al. 2004) and could respond similarly to the much more thoroughly studied model Drosophila. Further, the siRNA and JAK/STAT pathways are involved in cross-talk with one another in response to pathogenic invasion (Niu 2015), and the JAK/STAT has been implicated in viral immune response in the Hymenopteran Bombus terrestris, the buff-tailed bumblebee. In order to evaluate the involvement of the JAK/STAT in B. terrestris viral immune response, Niu, 2015 silenced the key JAK/STAT component Hop prior to inoculation with Israeli Acute Paralysis Virus (IAPV) and Slow Bee Paralysis Virus (SPBV). This study showed no significant relationship between IAPV titer compared to the control uninfected bees, but there was a significant increase in SPBV viral titer at two days postinfection, suggesting a temporal component to JAK/STAT signaling in viral immune response in B. terrestris (Niu 2015) (Supp Material [online only]).

Concluding Remarks
The immune response of social Hymenoptera to the challenge of the viral pathogens that infect them is a dynamic, multifaceted topic that has only recently begun to be teased apart. As such research efforts have progressed, the gravity and importance of innate immunity to economic biodiversity interests are being realized. Eusocial Hymenoptera are ecologically and economically significant both in detrimental and contributed capacities. Among these, the honey bee A. mellifera is one of the most economically beneficial insects in human agriculture, contributing billions of US dollars annually on a global scale (Brutscher et al. 2015, Popovska Stojanov et al. 2021). Despite the staggering importance role of the honey bee and conservation efforts to preserve them, upwards of 30% of United States colonies die off annually, with many of these losses attributed to Colony Collapse disorder (CCD) (Brutscher et al. 2015, McMenamin and Generisch 2015, McMenamin and Flenniken 2018). As this principal pollinator continues to be ravaged by viral disease, the necessity for further research on the innate immune system becomes increasingly apparent. On the opposite end of the interest spectrum are invasive ant species. Invasive ants are exceptionally successful, to the point of causing significant economic and biodiversity losses. With this in mind, the search for safe, effective, host-specific, and self-propagating biological control agents to combat invasive ants has led to the identification of a number of viral natural enemies that are considered promising biological control agents. Though viral natural enemy application is an exciting area of research, an understanding of host-agent interactions and immune response of host to pathogen is critical to biological control success. As the impact of viral pathogens infecting the eusocial insects becomes increasingly apparent, expanding upon the understanding of viral pathogen challenge becomes increasingly relevant to both research science and agriculture.

Immunity induced by even closely related pathogens is highly diverse and somewhat inconsistent across taxa, and the full implications of these responses may only be projected at present. Viruses could inhibit induction of certain pathways, and immune response to even closely related viral natural enemies can vary considerably across taxa. Also interesting is the observation that, particularly in ants, some pathways are effective against certain viruses but not others; some genes were positively correlated with viral challenge in Argentine ants, whereas others were negatively correlated. The insect immune response to viral pathogen invasion is an area of study that still provides copious opportunities for new research, particularly as it pertains to pathogen-based biological control approaches of pest insects. Further recent work has shone light on the roles of transmissible RNAs (Maori et al. 2019), transgenerational immune priming (Amiri et al. 2020), and heat shock proteins (McMenamin et al. 2020) in both social and innate immunity. There is undoubtedly much to be learned about immune response to viral pathogens in eusocial Hymenoptera; the future is bright for this exciting area of study.

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