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Weight of evidence evaluation of a network of adverse outcome pathways linking activation of the nicotinic acetylcholine receptor in honey bees to colony death

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Review

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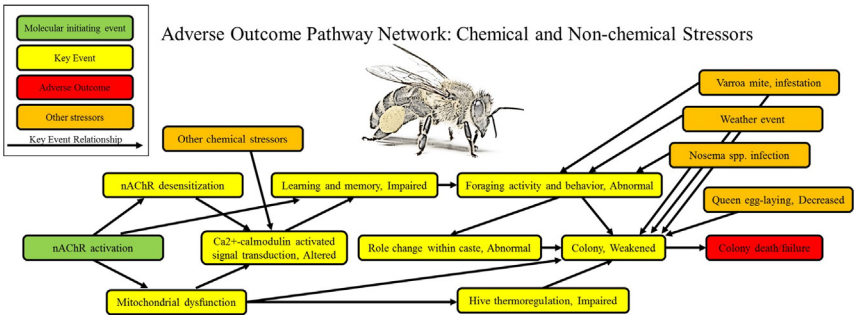
HIGHLIGHTS

- Six AOPs were developed describing perturbation of the honey bee nicotinic acetylcholine receptor leading to colony death.
- From weight of evidence evaluation, sufficient biological plausibility exists to link activation of nAChR to colony death.
- Uncertainties remain in the AOP descriptions, identifying knowledge gaps that can guide future research.
- These AOPs provide a foundation for future collaborations to understand chemical and non-chemical stressors on bee colonies.

GRAPHICAL ABSTRACT



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ABSTRACT

Ongoing honey bee (*Apis mellifera*) colony losses are of significant international concern because of the essential role these insects play in pollinating crops. Both chemical and non-chemical stressors have been implicated as possible contributors to colony failure; however, the potential role(s) of commonly-used neonicotinoid insecticides has emerged as particularly concerning. Neonicotinoids act on the nicotinic acetylcholine receptors (nAChRs) in the central nervous system to eliminate pest insects. However, mounting evidence indicates that neonicotinoids also may adversely affect beneficial pollinators, such as the honey bee, via impairments on learning and memory, and ultimately foraging success. The specific mechanisms linking activation of the nAChR to adverse effects on learning and memory are uncertain. Additionally, clear connections between observed impacts on individual bees and colony level effects are lacking. The objective of this review was to develop adverse outcome pathways (AOPs) as a means to evaluate the biological plausibility and empirical evidence supporting (or refuting) the linkage between activation of the physiological target site, the nAChR, and colony level consequences. Potential for exposure was not a consideration in AOP development and therefore this effort should not be considered a risk assessment. Nonetheless, development of the AOPs described herein has led to the

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identification of research gaps which, for example, may be of high priority in understanding how perturbation of pathways involved in neurotransmission can adversely affect normal colony functions, causing colony instability and subsequent bee population failure. A putative AOP network was developed, laying the foundation for further insights as to the role of combined chemical and non-chemical stressors in impacting bee populations. Insights gained from the AOP network assembly, which more realistically represents multi-stressor impacts on honey bee colonies, are promising toward understanding common sensitive nodes in key biological pathways and identifying where mitigation strategies may be focused to reduce colony losses.

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1. Introduction

The increase in colony loss among managed honey bees (*Apis mellifera*) and population declines in some non-*Apis* bees observed in recent years could have significant economic and ecological implications. Approximately 85% of all flowering plants are pollinated by animals (Ollerton et al., 2011) with managed honey bee and non-*Apis* bee species pollinating, respectively, over $19 billion and $3 billion worth of crop plants in the United States in 2010 (https://www.fws.gov/pollinators/; accessed October 2016; Losey and Vaughan, 2006). Along with other pollinators, bees play a crucial role in maintaining plant biodiversity and food supplies for humans, livestock, and wildlife (Potts et al., 2010). Due to the widespread consequences that could occur if current bee losses are not reduced, it is necessary to objectively evaluate potential contributing factors that may lead to declines in honey bee colonies. Numerous influences have been proposed, including poor honey bee keeping practices, pathogens and parasites, loss of habitat, climate change, and pesticides (Potts et al., 2010). Although recent evidence suggests that multiple stressors contribute to colony loss by acting in combination, chemical pesticides are one such factor receiving significant attention. Among pesticides, neonicotinoids have risen to be one of the most widely used insecticide classes. Given their persistence in the environment, uptake and systemic distribution by plants, and high toxicity to bees, neonicotinoids have received heightened attention by the general public, and both the scientific and regulatory communities, for their possible role in bee colony losses (White House, 2015; US Environmental Protection Agency, 2016; Godfray et al., 2015; European Food Safety Authority, 2015).

Neonicotinoids, which are synthetic derivatives of nicotine, are an important class of insecticides used worldwide to control a number of agriculturally destructive plant-chewing, -piercing, and -sucking insects (Tomizawa and Casida, 2005). They act as nicotinic acetylcholine receptor (nAChR) agonists and cause neurotoxicity in target pests (Matsuda et al., 2001). In the US there are eight currently registered active ingredients, including imidacloprid (earliest neonicotinoid registered in 1994), thiamethoxam, acetamiprid, clothianidin, thiacloprid, nithiazine, nitenpyram (registered for veterinary applications), and dinotefuran (registered in 2012) (Fairbrother et al., 2014). Due to field efficacy, flexibility in application methods, low vertebrate toxicity (compared to alternative insecticides, such as organophosphates), and resilience to the development of target pest resistance, neonicotinoids have had a prominent role in the insecticide market, surpassing two billion dollars (U.S. currency) in 2009 global sales (Jeschke et al., 2011; Tomizawa and Casida, 2005). Therefore, driven in part by the assertion that insecticides, such as neonicotinoids, may play a role in honey bee colony losses, and further by the increased usage of neonicotinoids for crop protection, a significant amount of scientific investigation has ensued over the past two decades to enhance understanding of the toxicity of these chemicals to both targeted and beneficial insects, particularly honey bees and bumble bees (*Bombus* spp.). With the growing body of literature focused in this area, there is an opportunity to organize empirical support using an adverse outcome pathway (AOP) framework, as a means to systematically evaluate the biological plausibility and empirical data in a weight of evidence (WoE) evaluation to understand the potential linkage between activation of the nAChR (i.e., molecular initiating event) and subsequent key events across multiple levels of biological organization leading to population-level impacts on honey bee colonies (Ankley et al., 2010).

The AOP construct has increasingly been used to capture existing knowledge establishing links between chemical perturbation of a biomolecule (e.g., the nAChR), termed the molecular initiating event (MIE), and measurable biological changes spanning multiple levels of biological organization that culminate in an adverse outcome (AO) considered significant for risk assessment, typically at the individual (e.g., individual bee) or population (e.g., failure of multiple colonies) level (Ankley et al., 2010). Adverse outcome pathways are represented as sequences of key events (KEs), which describe essential and measurable changes in the biological state, and key event relationships (KERs), which define the nature of and evidence for a particular relationship between a pair of KEs (Villeneuve et al., 2014). The systematic assessment of WoE provides a means to characterize the relative level of scientific support and confidence in the relationships linking an upstream KE to a downstream KE and to the overall AOP (Becker et al., 2015). Understanding the degree of confidence in the AOP is critical when considering the potential applications. Because AOPs are considered "living" documents, it is anticipated that as new experiments are published, AOPs and corresponding WoE analyses will evolve accordingly (Villeneuve et al., 2014). Weight of evidence analysis can help identify the points in the AOP (e.g., specific KERs) for which additional evidence could best support intended or desired applications.

There is utility for an AOP at any stage of development. Even a putative pathway which lacks detail or strong empirical evidence can, for example, aid in identifying research gaps. At the other end of the spectrum, well-defined AOPs that incorporate quantitative understanding of the biology allowing for extrapolation from one end of the pathway (e.g., laboratory-based studies) to the other (e.g., field-based studies) can support a wide range of regulatory applications (Wittwehr et al., 2016). Through constructing AOPs and associated WoE evaluations relevant to chemical stressors on honey bee populations, a better understanding of the state of the science and degree of confidence linking chemical perturbation of a pathway to colony loss can be elucidated.

In developing AOPs relevant to honey bee colony survival, it is important to recognize the unique social and behavioral underpinnings that regulate these eusocial insects, which may impact their ability to thrive upon exposure to natural and/or anthropogenic stressors. Due to the highly defined and interactive nature of honey bee colonies, they sometimes are referred to as a "superorganism" (Seeley, 1989). The honey bee colony, made up of 20,000–40,000 individuals, consists of three castes, the queen, workers (sterile females), and drones (males), all of which perform specific tasks, at relatively fixed life stages, dividing labor among tens of thousands of individuals (as reviewed by Page and Peng, 2001). The primary role of the highly fecund queen is laying eggs throughout the spring and summer months (Hoover et al.,

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2003). The development period from egg to adult for queens is 14 days with a typical adult lifespan of 2–5 years, although queens are generally replaced after 1–2 years in commercial operations due to lower quantity and quality of brood produced (Sammataro and Avitabile, 1998).

The role of all other female worker bees is maintaining normal colony functions, including queen and brood care, grooming and other hygienic hive tasks, guarding the nest, foraging, processing and storing of nectar and pollen, and hive thermoregulation (Sagili et al., 2011; Elekonich and Roberts, 2005). The typical developmental process from egg to adult for worker bees is 21 days, followed by another 21 days of in-hive activities prior to taking on foraging roles (Winston, 1987; Seeley, 2014). Although these tasks are primarily age dependent, there is plasticity within the worker sub-caste, whereby they can modify their roles as necessary for colony survival (Münch et al., 2008). Worker bees emerging in the spring and summer may live only 5–6 weeks, whereas those emerging in the autumn and maintaining the hive over winter, can live 6–8 months or more (Amdam and Omholt, 2002). The role of male drones is to fertilize a queen for propagation of the hive. Drones reach adulthood after ~24 days of development and sexual maturity at 16 days old. Drones then begin searching for a queen to fertilize but become less efficient for mating after 28 days of age (Rhodes, 2002). Typically drones live for ~59 d (as reviewed by Page and Peng, 2001). Drones are produced only for a short period during the summer and die following successful mating or upon eviction from the hive in autumn months in preparation for colony over-wintering (Free and Williams, 1975).

In the fall, queen bees halt brood production, food sources become scarce, and workers cease foraging activity, so the colony relies solely on stored food to meet energy demands (Seeley and Visscher, 1985). The mass of workers participates in clustering and shivering thermogenesis to ensure survival of the queen, brood, and colony during the winter months (Stabentheiner et al., 2003). Winter is a critical period, and the colony must be of sufficient health and size, with necessary sustenance to withstand the stress of colder temperatures in order to emerge in the spring equipped for resuming foraging and reproductive activities.

Declines in honey bee colony health and high colony losses have been reported by beekeepers over the last decade at rates, ranging from 23 to 41% (Lee et al., 2015b; Spleen et al., 2013; Steinhauer et al., 2014; Seitz et al., 2015; vanEngelsdorp et al., 2010; vanEngelsdorp et al., 2011; vanEngelsdorp et al., 2012). These levels are noticeably higher than the economically sustainable losses of about 15% reported by the US beekeepers (Steinhauer et al., 2014). Many stressors, including pests, pathogens, agricultural intensification, poor nutrition, improper management practices, failing queens, and pesticide exposure have been identified as contributing to continued high losses (Evans et al., 2009; Seitz et al., 2015). With the importance of honey bee pollination, it is imperative to capture available data to assess the degree to which a linkage may (or may not) exist between chemical stressors, declines in bee health, and/or honey bee colony losses.

The purpose of the present review and analysis was to utilize the AOP framework to assemble available evidence related to the possible linkage of nAChR activation and adverse effects on the honey bee colony as a super-organism. An AOP network connecting six linear AOPs was constructed, providing a basis to begin evaluating which nodes may be impacted by multiple stressors. Through the process of organizing available information from the literature using the AOP conceptual framework, and conducting a WoE evaluation of key knowledge gaps, important research needs were identified. Given the breadth of available literature on these topics, comprehensive development of all plausible AOPs linking nAChR activation to colony death was not practical. Therefore, keeping in mind that AOPs are living documents, and that collaboration is essential to the development of AOPs (Villeneuve et al., 2014), we envision that the AOP network described herein could provide the foundation for future communication and collaborations to more thoroughly describe the impacts of both chemical and non-chemical stressors on honey bee colonies.

It is critical to note that the various AOPs described below assume that chemical perturbation of a given MIE occurs at levels (i.e., dose) sufficient to produce an AO. Our analysis does not explicitly include consideration of quantitative environmental exposure of bees to any particular neonicotinoid mentioned herein, nor absorption, distribution, metabolism, or elimination considerations that may be pertinent to a given chemical/exposure scenario. Within the AOP descriptions, the only mention of chemical concentrations are those reported in the cited studies. Consequently, this analysis should not be considered a risk assessment in that exposure is not incorporated into the AOP development/description (Villeneuve et al., 2014). The fact that a biologically and empirically supported linkage between an insecticide-mediated MIE and colony death exists, does not necessarily imply real world exposure conditions are sufficient to evoke the level of perturbation needed to drive the pathway to the AO.

2. Development of adverse outcome pathways

Based on review of the published literature, six linear, putative, AOPs were developed to describe the mechanism(s) linking activation of the nAChR to the population level AO of regulatory concern, i.e., honey bee colony death or failure (Fig. 1). The MIE of nAChR activation and the AO of colony death/failure were shared among all six AOPs. Eight intermediate KEs were identified, including nAChR desensitization, mitochondrial dysfunction, altered Ca^{2+}-calmodulin activated signal transduction, impaired learning and memory, abnormal foraging activity and behavior, abnormal role change within caste, impaired hive thermoregulation, and weakened colony (as defined by reduced brood or numbers of adults, low food stores, and/or increased mortality). In accordance with best practices for AOP development, and to facilitate future collaboration and expansion of the AOP network, KE descriptions were developed to allow for reusability among the AOPs (Villeneuve et al., 2014) and have been entered into the publically available AOP development platform, the Adverse Outcome Pathway Wiki (AOPWiki; https://aopwiki.org/; Supplemental Materials, Table S1). Seven of the eight intermediate KEs described in this manuscript are used in more than one AOP, with impaired hive thermoregulation being the exception. However, it is anticipated that other chemical and non-chemical stressors will impact hive thermoregulation, so this KE description is likely to be important for future AOP development.

Key events directly downstream of nAChR activation include either nAChR desensitization (Fig. 1, KE in AOP1 and AOP2) or mitochondrial dysfunction (Fig. 1, KE in AOP3, AOP4, AOP5, and AOP6), both of which can lead to three shared subsequent KEs: altered Ca^{2+}-calmodulin activated signal transduction, followed by impaired learning and memory, and then abnormal foraging activity and behavior. From the KE of abnormal foraging activity and behavior, both a direct link to the downstream KE of weakened colony is described, as well as alternative pathways (Fig. 1, AOP2 and AOP 4) in which an additional KE of abnormal role change within caste (e.g., precocious foraging by young hive bees) exists between these KEs.

Another AOP was also developed that directly links mitochondrial dysfunction to impaired hive thermoregulation, which may impact brood development and production or susceptibility to pests and pathogens (e.g., *Varroa destructor* mite and the microsporidian *Nosema ceranae*) and lead to weakened colony and further colony death/failure. Finally, an AOP was developed directly linking mitochondrial dysfunction, described in part by disruption of mitochondria responsible for generating the energy that powers most cell functions, to weakened colony. Therefore, depending on the AOP being described, four different upstream KEs (Fig. 1, KE2, KE5, KE6, or KE7) were linked to weakened colony, which is then linked to colony death/failure, where colonies have complete loss of adults or unsustainably low numbers of bees and cannot perform normal colony functions such as brood care or resource acquisition and will not survive winter months. Colony death/failure is the downstream anchor, and AO in all of the AOPs.

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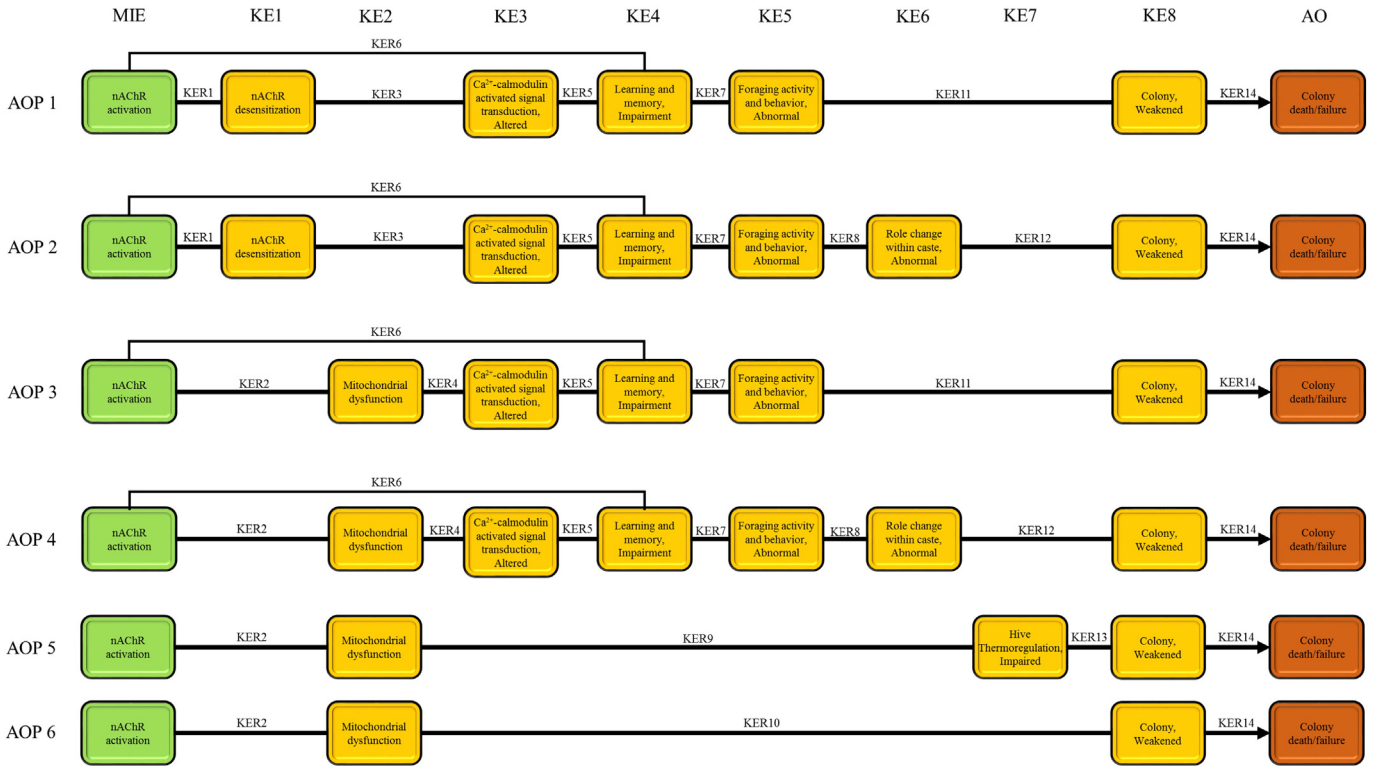

Fig. 1. Six linear adverse outcome pathways (AOPs) describing the key events (KE) linking the molecular initiating event (MIE) of nicotinic acetylcholine receptor (nAChR) activation to the adverse outcome (AO) of regulatory concern, honey bee colony death/failure. Green boxes depict the molecular initiating event, yellow boxes represent intermediate KEs, and the red boxes indicate the adverse outcome. Multiple events are shared by more than one AOP. Solid black connecting lines represent key event relationships (KERs). The AOP, KE, and KER numbers are referenced throughout the text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

From the six AOPs, fourteen KERs linking upstream KEs to downstream KEs were described and used to evaluate WoE. Three primary considerations are taken into account for WoE assessment for AOPs: biological plausibility, evidence for essentiality of the KEs, and empirical support for the KERs (Becker et al., 2015). Biological plausibility refers to the structural or functional relationship between KEs as defined, primarily, by the understanding of "normal" unperturbed biology. For example, if it is known that binding of a neurotransmitter to its cognate receptor is required for downstream neuronal signaling, then it is plausible that antagonism of the receptor could impair downstream neuronal signal transduction (Fig. 1; KER3), whether that perturbation had been specifically tested or not. Evidence for a second WoE consideration, essentiality, refers to demonstration that if a given KE is prevented, other downstream KEs in the sequence depicted for a given AOP also do not occur. Evidence for essentiality effectively establishes an upstream KE as the cause for the changes in subsequent downstream KEs. Essentiality can be described as 'strong', 'moderate', or 'weak' based on the evidence for the causal importance of the KE in the sequence (Table 1). Classic types of experiments used to establish essentiality include gene knock down or over-expression, co-treatment with pharmaceuticals with opposing action, or stop-exposure and recovery studies. Many of the KER descriptions herein describe studies employing pharmaceutical manipulation or knockdown experiments that provided the evidence for 'strong' essentiality (Table 1). Finally, empirical support refers to specific evidence that shows that when an upstream KE occurs, the downstream KE tends to occur as well, and does so in a manner that is concordant with the assumption of a causal relationship. When data are abundant and consistent with expected patterns, the empirical support is viewed to be strong. When there are unexplained inconsistencies with expected patterns, the empirical support may be weak even if there are considerable data available. Among the three types of considerations, biological plausibility is weighted most heavily in assessing overall WoE or confidence (OECD, 2016). When a structural or functional relationships between KEs is well understood, a fairly modest amount of direct empirical evidence can provide strong support for a KER. In the end, the stronger the support for causal relationships between KEs in the AOP, the stronger confidence in extrapolating along the AOP. Guiding questions for assigning "strong, moderate, or weak" designations for each of these elements of WoE for AOPs have been developed (OECD, 2016) and were applied in the present work.

3. AOP descriptions

The following section describes relevant chemical initiators known to act on the nAChR with subsequent sections focused on descriptions of KEs, KERs, and associated WoE for the six distinct AOPs. In accordance with previously defined guidance for AOP development, which identify KEs as measurable changes in biology, methods for assessing each KE are also identified (Table 2; Villeneuve et al., 2014). The KE descriptions in each section typically include: (a) an outline of the "normal" biology occurring at the specified level of biological organization, (b) support for biological plausibility linking one KE to another, and (c) when available, empirical evidence supporting those KERs. Due to the extensive amount of literature available for describing the WoE to link KEs, the current evaluation was not meant to be comprehensive, but sought to capture pertinent literature relevant to the relationships. We anticipate that WoE for any KER will continue to evolve as new studies are conducted and additional expertise is applied. The WoE described below focuses on KERs that were identified as 'moderate' or 'weak' (Table 3). Those KERs determined to fulfill requirements of 'strong' biological plausibility and empirical evidence are not explicitly discussed, as the strength in the WoE is either considered dogma or apparent from the biological plausibility and evidence outlined in the KER description. Further, all KEs were identified as having 'strong' essentiality, with the exception of abnormal role change within caste (Table 1). Therefore, essentiality

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Table 1
Assignment of essentiality to key events.

[a]Adverse outcome pathway	Key event	Support for essentiality
1	nAChR desensitization	Strong
	Ca^{2+}-calmodulin activated signal transduction, Altered	Strong
	Learning and memory, Impairment	Strong
	Foraging activity and behavior, Abnormal	Strong
	Colony, Weakened	Strong
2	nAChR desensitization	Strong
	Ca^{2+}-calmodulin activated signal transduction, Altered	Strong
	Learning and memory, Impairment	Strong
	Foraging activity and behavior, Abnormal	Strong
	Role change within caste, Abnormal	Cannot establish
	Hive thermoregulation, Impaired	Strong
	Colony, Weakened	Strong
3	Mitochondrial dysfunction	Strong
	Ca^{2+}-calmodulin activated signal transduction, Altered	Strong
	Learning and memory, Impairment	Strong
	Foraging activity and behavior, Abnormal	Strong
	Colony, Weakened	Strong
4	Mitochondrial dysfunction	Strong
	Ca^{2+}-calmodulin activated signal transduction, Altered	Strong
	Learning and memory, Impairment	Strong
	Foraging activity and behavior, Abnormal	Strong
	Role change within caste, Abnormal	Cannot establish
	Colony, Weakened	Strong
5	Mitochondrial dysfunction	Strong
	Hive thermoregulation, Impaired	Strong
	Colony, Weakened	Strong
6	Mitochondrial dysfunction	Strong
	Colony, Weakened	Strong

[a] AOP number assignment (1–6) assigned from Fig. 1.

Table 2
Methods to measure key events and identification of key data gap.

Key event	Methods for measure or observation
Nicotinic acetylcholine receptor, Activation	• Radiolabeled nAChR agonists, (e.g., [3H] imidacloprid) or nAChR subunit specific antibodies to detect location and subunit composition of nAChR • Ligand competition studies evaluating [3H] agonist displacement to determine ligand affinities to the nAChR • Whole-cell voltage clamp electrophysiological measurements with agonists to measure nAChR activation
Nicotinic acetylcholine receptor, Desensitization	• Electrophysiological characterization for investigation of desensitization. Patch-clamp, number of channel openings per unit time • Immunoblotting to determine phosphotyrosine content of purified nAChR
Mitochondrial dysfunction	• Oxygen consumption rates as a measure of mitochondrial respiration • ATP quantification using the firefly luciferin-luciferase assay system
Ca^{2+}-calmodulin activated signal transduction, Altered	• Fluorescent Ca^{2+} imaging in cells expressing nAChR for evaluation of Ca^{2+} levels entering individual nAChR-mediated ion channels • Western blotting and kinase assays can be used to evaluate ERK1/2 phosphorylation and activity • Activation of CREB/CRE transcription • Pharmacological inhibition of pathway
Learning and memory, Impaired	• Proboscis Extension Assay • Training to forage specific site with or without conditioned stimulus
Foraging activity and behavior, Abnormal	• Radio-frequency identification tagging technology to track the frequency and duration of individual foraging events, flight time, foragers homing ability, duration of time spent at a feeder, and duration between feeding • Video tracking software for measures of total distance traveled and time spent in social interaction • Weigh bee-collected pollen from hive entrance trap • Pollen load can also be assessed by scoring the size of amount of pollen in the forager's corbiculae (pollen basket) relative to the size of the worker bee • Nectar loads from individual forager bees can be measured with a pocket refractometer after inducing regurgitation • Video foragers returning to hive and measure waggle dance circuits performed • Food storage can be measured by visual inspection or digital imaging of the combs with the objective to estimate the percent of cells filled with nectar (uncapped), honey (capped), or pollen
Role change within caste, Abnormal	• Age of first forage • Hypopharyngeal gland development in forage bees that revert to hive bees
Hive thermoregulation, Impaired	• Infrared thermography to measure the body surface temperature of bees • Microcalorimetric determinations of heat production • Number of bees in cluster
Colony, Weakened	• Count number of adult bees, presence of sealed and open brood, assess amount of food stores by visual method or by weighing, assess presence/absence of pests and disease, evaluate egg laying patterns of queen • Brood care behavior can be evaluated by filming the brood nest and then recording nursing frequency, total nursing period per hour, and average duration of nursing episodes for individual cells • Cannibalism of brood can be detected by mapping eggs, larvae and pupae present on brood frames and noting developmental stages for each individual, then inspecting daily for missing larvae • Assess health of bee: dry weight, muscle development, protein content

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is discussed in the KER description for abnormal foraging activity and behavior leading to abnormal role change within caste.

3.1. Chemical initiators

The nicotinoids and neonicotinoids are both agonists of the nAChR (Tomizawa and Casida, 2003); however, neonicotinoids are the primary chemicals considered in the AOPs relevant to bees.

The potency of a nAChR agonist is dependent on the receptor subunit composition, structurally important amino acid residues at the binding site, and the ionization status of the chemical at physiological pH (Tomizawa and Casida, 2003; Dani and Bertrand, 2007). For example, nicotine is a classical vertebrate nAChR agonist; however, it has relatively low affinity (and insecticidal activity) for the invertebrate nAChR. Due to ionization, nicotine is poor at passing though the ion-impermeable barrier surrounding the insect central nervous system (CNS; Tomizawa and Casida, 2003). Conversely, non-ionizable neonicotinoids readily translocate into the insect CNS and have high affinity for the nAChR (e.g., *Drosophila* nAChR IC_{50} 4.6 nM imidacloprid), with limited or no binding activity to vertebrate nAChR (Tomizawa and Casida, 2003). Various studies have demonstrated that similarities and differences in key amino acid residues in the ligand binding domain across species can lead to structural and binding site differences that dictate chemical interaction with the receptor (Dani and Bertrand, 2007; Matsuda et al., 2009; Tomizawa and Casida, 2009; Jones and Sattelle, 2010; LaLone et al., 2016). Due to the intended insecticidal action of neonicotinoids, a growing number of studies have been conducted to evaluate potential adverse effects in non-target species such as honey bees exposed to neonicotinoids, particularly imidacloprid, clothianidin, and thiamethoxam. Some of the results of these studies are included in subsequent AOP descriptions.

3.2. MIE: nicotinic acetylcholine receptor activation

Nicotinic acetylcholine receptor activation is the MIE in the developed AOPs (Fig. 1). Nicotinic acetylcholine receptors belong to the cys-loop superfamily of ligand-gated ion channels, responsible for rapid neurotransmission (Karlin, 2002). In insects nAChR have signaling roles in nervous systems and neuromuscular junctions and other cells (Jones and Sattelle, 2010; Lindstrom, 2003). Under normal conditions the endogenous neurotransmitter, acetylcholine (ACh), attaches to the ligand binding domains on the extracellular region of the pentameric nAChR. This initiates a conformation change that promotes the influx and efflux of calcium (Ca^{2+}) and extracellular sodium and intracellular potassium ions, respectively, to create the action potential necessary for synaptic signaling (Jones and Sattelle, 2010). Activation of the nAChR, by natural or synthetic agonists, and subsequent involvement in neurotransmission is well established. Although the nAChR is conserved across vertebrates and invertebrates, the diverse composition and assembly of α-(containing two adjacent cysteine residues important in ACh binding) and non α-(lacking the cysteine residues) subunits confer diverse functional architecture and, therefore, toxicological responses (Jones and Sattelle, 2010).

Table 3
Relationships among key events and the adverse outcome.

[a]Adverse outcome pathway	Key event	Description	Triggers	Weight of evidence
1	nAChR activation	Leads to	nAChR desensitization	Moderate
	nAChR activation	Leads to	Learning and memory, Impairment	Moderate
	nAChR desensitization	Leads to	Ca^{2+}-calmodulin activated signal transduction, Altered	Weak
	Ca^{2+}-calmodulin activated signal transduction, Altered	Leads to	Learning and memory, Impairment	Strong
	Learning and memory, Impairment	Leads to	Foraging activity and behavior, Abnormal	Strong
	Foraging activity and behavior, Abnormal	Leads to	Colony, Weakened	Strong
	Colony, Weakened	Leads to	Colony death/failure	Strong
2	nAChR activation	Leads to	nAChR desensitization	Moderate
	nAChR activation	Leads to	Learning and memory, Impairment	Moderate
	nAChR desensitization	Leads to	Ca^{2+}-calmodulin activated signal transduction, Altered	Weak
	Ca^{2+}-calmodulin activated signal transduction, Altered	Leads to	Learning and memory, Impairment	Strong
	Learning and memory, Impairment	Leads to	Foraging activity and behavior, Abnormal	Strong
	Foraging activity and behavior, Abnormal	Leads to	Role change within caste, Abnormal	Moderate
	Role change within caste, Abnormal	Leads to	Colony, Weakened	Weak
	Colony, Weakened	Leads to	Colony death/failure	Strong
3	nAChR activation	Leads to	Mitochondrial dysfunction	Weak
	nAChR activation	Leads to	Learning and memory, Impairment	Moderate
	Mitochondrial dysfunction	Leads to	Ca^{2+}-calmodulin activated signal transduction, Altered	Strong
	Ca^{2+}-calmodulin activated signal transduction, Altered	Leads to	Learning and memory, Impairment	Strong
	Learning and memory, Impairment	Leads to	Foraging activity and behavior, Abnormal	Strong
	Foraging activity and behavior, Abnormal	Leads to	Colony, Weakened	Strong
	Colony, Weakened	Leads to	Colony death/failure	Strong
4	nAChR activation	Leads to	Mitochondrial dysfunction	Weak
	nAChR activation	Leads to	Learning and memory, Impairment	Strong
	Mitochondrial dysfunction	Leads to	Ca^{2+}-calmodulin activated signal transduction, Altered	Moderate
	Ca^{2+}-calmodulin activated signal transduction, Altered	Leads to	Learning and memory, Impairment	Strong
	Learning and memory, Impairment	Leads to	Foraging activity and behavior, Abnormal	Strong
	Foraging activity and behavior, Abnormal	Leads to	Role change within caste, Abnormal	Moderate
	Role change within caste, Abnormal	Leads to	Colony, Weakened	Weak
	Colony, Weakened	Leads to	Colony death/failure	Strong
5	nAChR activation	Leads to	Mitochondrial dysfunction	Weak
	Mitochondrial dysfunction	Leads to	Hive thermoregulation, Impaired	Weak
	Hive thermoregulation, Impaired	Leads to	Colony, Weakened	Strong
	Colony, Weakened	Leads to	Colony death/failure	Strong
6	nAChR activation	Leads to	Mitochondrial dysfunction	Weak
	Mitochondrial dysfunction	Leads to	Colony, Weakened	Weak
	Colony, Weakened	Leads to	Colony death/failure	Strong

[a] AOP number assignment (1–6) assigned from Fig. 1.

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Interestingly, in mammals, nAChRs have been shown to be expressed in the outer membranes of mitochondria, with subtypes having tissue-specific locations (Gergalova et al., 2012; Gergalova et al., 2014; Kalashnyk et al., 2012; Lykhmus et al., 2014). For example, in mice, brain and liver mitochondria typically express α7β2 and α4β2 nAChRs, whereas lung predominantly express α3β2 nAChRs (Lykhmus et al., 2014). Therefore, evidence of mitochondrial nAChRs in mammals provides biological plausibility that these receptors may be expressed in invertebrate mitochondria as well. However, localization of nAChR in the mitochondrial membranes of invertebrates has not, to our knowledge, been investigated. This represents a knowledge gap that may warrant future research.

3.3. KE1: desensitization of nicotinic acetylcholine receptor

A KE directly downstream of nAChR activation is desensitization of the nAChR. This KE is shared in two AOPs (Fig. 1, AOP1 and AOP2). Directly downstream of this KE is the KE of altered Ca^{2+}-calmodulin activated signal transduction. Desensitization of the nAChR occurs at the molecular level of biological organization.

3.3.1. Normal biology

Upon prolonged and repeated exposure to a nAChR agonist, desensitization may occur. Desensitization is characterized by an initial opening of the ion channel and ion exchange across the cell membrane followed by rapid channel closure and inactivity, effectively inhibiting neurotransmission (Quick and Lester, 2002). Further, inhibition of nAChR activity from desensitization can lead to an up-regulation in nAChR expression, termed pharmacological chaperoning (Srinivasan et al., 2012; Flores et al., 1992; Marszalec et al., 2005). Exposure to imidacloprid and thiamethoxam for 72 or 48 h, respectively was shown to significantly increase transcriptional abundance of nAChRα1 subunit in the honey bee brain (Christen et al., 2016). In the desensitized state, nAChR receptors have high affinity for the agonist and therefore establish a blockade to subsequent agonist binding (Ochoa et al., 1989). It has been demonstrated that recovery from nAChR desensitization occurs (though not always complete) upon removal of the agonist (Ochoa et al., 1989). However, the speed of recovery is dependent on the concentration and duration of exposure to the agonist, with longer exposures typically resulting in slower recovery times (Quick and Lester, 2002). In fact, loss of functional nAChR channels has been reported in neuronal cell line PC12 (rat adrenal gland pheochromocytoma tumor) upon prolonged exposure to carbachol, a cholinergic agonist (Simasko et al., 1986).

Phosphorylation of nAChR subunits is another factor that regulates the rate of desensitization and subsequent recovery. Nicotinic acetylcholine receptor subunits possess phosphorylation sites for cAMP-dependent protein kinase A (PKA), protein kinase C (PKC), calcium-calmodulin-dependent protein kinase (CaM kinase) and endogenous protein tyrosine kinase (Hopfield et al., 1988; Thany et al., 2007). Evidence suggests that phosphorylation of nAChR subunits regulate the rate of desensitization, with the greater number of phosphotyrosines indicative of rapid recovery from desensitization (Hopfield et al., 1988; Thany et al., 2007).

3.4. KER1: nAChR activation leads to desensitization

The KER linking nAChR activation to desensitization is involved in two AOPs (Fig. 1; AOP1 and AOP2) and is supported by both biological plausibility and empirical information. The WoE assessment for this KER examines the presence of nAChRs in honey bee neurons and whether activation of the nAChR can lead to desensitization.

3.4.1. Consideration of biological plausibility and empirical support

The first draft of the honey bee genome became available through the efforts of the Honey Bee Genome Sequencing Consortium (2006), and has provided valuable insights on evolution and comparisons between species. The honey bee has 11 genes that encode nAChR subunits - nine α and two β subunits (Jones et al., 2006), consistent with the condensed number of genes seen in other insects compared to vertebrates (Tomizawa and Casida, 2001). The primary location of insect nAChRs is the brain. In honey bees, nAChRs have been identified in Kenyon cells located on mushroom bodies and antennal lobes, both involved in olfactory learning (Deglise et al., 2002; Dupuis et al., 2011).

It has been demonstrated in various models that nAChR agonism does indeed lead to desensitization. For example, upon exposure of human α7 nAChR expressed in African clawed frog (*Xenopus laevis*) oocytes to classical nAChR agonists, including nicotine, Briggs and McKenna (1998) showed that even weak or low concentrations of an agonist could act as more potent inhibitors than activators of the receptor through desensitization. Further, in another example measuring current across the neuron and activity of the natural nAChR ligand and ACh neurotransmitter, Zwart et al. (1994) demonstrated that six nAChR agonists induced nAChR-mediated inward ionic current, and that their continued presence significantly blocked ACh-induced inward current in whole-cell voltage-clamped African locust (*Locusta migratoria*) thoracic ganglion neurons. In that study, it was shown that concentrations of 0.1 μM and 10 μM imidacloprid induced ACh-inward current with peak amplitudes of 4% and 30%, respectively (Zwart et al., 1994). Continued exposure to 0.1 μM imidacloprid led to desensitization that reduced the amplitude of 1 mM ACh-induced inward current by 73%; whereas, continued exposure to 10 μM imidacloprid completely blocked inward current indicting that the potency to block the ACh-induced ion current was greater than the potency to induce inward current (Zwart et al., 1994).

Specific evidence of desensitization exists in honey bees as well. Exposure of cultured Kenyon cells from honey bee brains to imidacloprid yielded partial nAChR agonist activity, eliciting 36% of the ACh-induced current and causing desensitization of the receptor after prolonged (16 s) exposure (Deglise et al., 2002). Further, when 10^{-5} M imidacloprid was co-applied with ACh, the mean amplitude of ACh-induced currents was significantly lowered (64%) compared to ACh co-application with saline, thereby providing evidence that imidacloprid antagonized the ACh-induced receptor activation by out-competing ACh for the same binding site (Deglise et al., 2002). Interestingly, an antagonist of the nAChR (mecamylamine) demonstrated similar properties, likely affecting neurotransmission, in that direct injection into the brain hemolymph of honey bee was shown to not only impair olfactory learning but, in patch-clamp experiments with cultured Kenyon cells, completely block the ACh-induced current (Lozano et al., 1996; Wüstenberg and Grünewald, 2004).

Recovery from desensitization depends on the availability of phosphorylation sites on the nAChR subunits and the number of phosphotyrosine residues. Mutation of key PKC phosphorylation sites on the rat α4 nAChR subunit expressed in *Xenopus* oocytes resulted in impaired recovery from deep desensitization (Fenster et al., 1999). Further inhibition of PKC or knockout of PKC in a mouse model (Prkce −/−) also led to impaired recovery from desensitization (Lee et al., 2015a). Phosphorylation sites on nAChR subunits as well as PKC isozymes continue to be identified. Cross species differences in those sites may contribute to the differences in sensitivity to various chemicals that act on the nAChR (Hug and Sarre, 1993). Demonstration that perturbation to PKC can impact recovery from desensitization is an important piece of evidence, describing a potential feedback loop linking the downstream KE of altered Ca^{2+}-calmodulin activated signal transduction back to desensitization (see Fig. 2; step 6). Kinases phosphorylate nAChR subunits, indicating that disruption of downstream signaling could further impact nAChR desensitization status.

3.4.2. Weight of evidence for KER1

In considering the KER linking activation of the nAChR to desensitization, the WoE was classified as 'Moderate' (Table 3). Desensitization

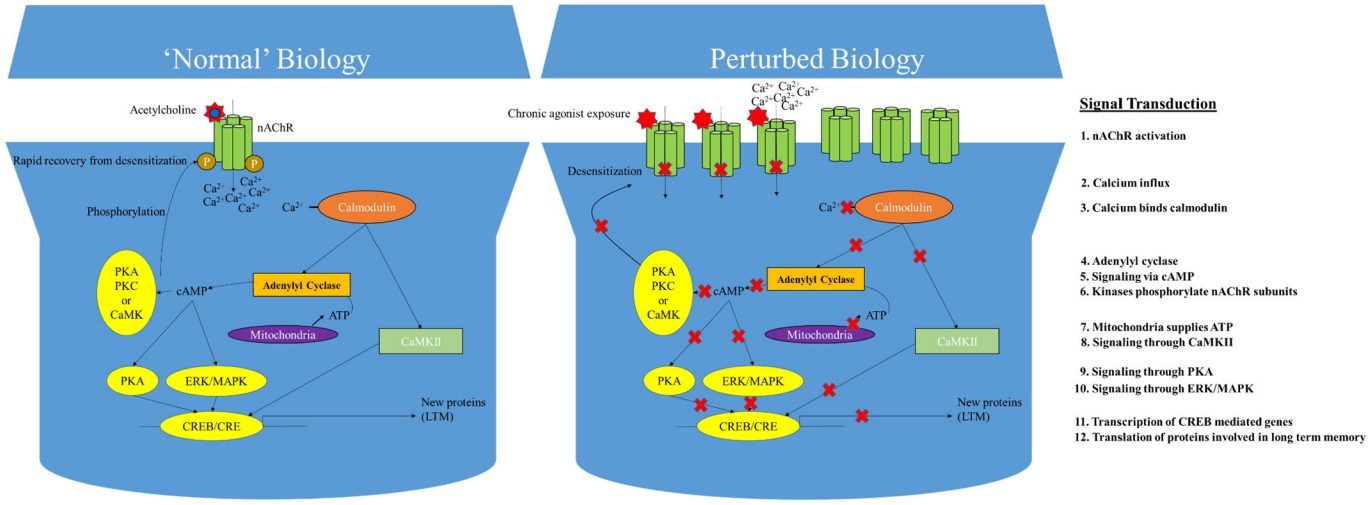

Fig. 2. Illustration of the mechanism linking activation of the nicotinic acetylcholine receptor (nAChR) to altered Ca^{2+}-calmodulin activated signal transduction (MIE, KE1, KE2, and KE3 from Fig. 1). Steps 1–12 describe the various components of the signaling pathway in each figure. (Left figure) Depiction of the 'normal' biology for neuronal signaling at the synapse via the nAChR leading to the production of new proteins involved in long term memory and (Right figure) an illustration of the same pathway perturbed by a chemical stressor via prolonged exposure to a nAChR agonist. Red x represents parts of the pathway where perturbation can lead to impacts on memory. Further, if phosphorylation of nAChR subunits is impaired, recovery from desensitization is also impeded. Abbreviations: nAChR, nicotinic acetylcholine receptor; Ca^{2+}, calcium; ATP, adenosine triphosphate; CaMKII, Ca^{2+}-calmodulin kinase II; cAMP, 3′5′-adenosine monophosphate; PKA, protein kinase A; PKC, protein kinase C; CaMK, Ca^{2+}-calmodulin kinase; ERK, extracellular signal-regulated protein kinase; MAPK, mitogen-activated protein kinase; CREB, cAMP response element binding protein; and CRE, cAMP response element. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

is a well-studied biological function that occurs upon activation of ligand-gated ion channels, such as the nAChR, with prolonged or repeated exposure to variable concentrations (typically low) of agonist; thus, biological plausibility of activation leading to desensitization is quite strong. However, there are relatively significant uncertainties associated with desensitization of the insect neuronal nAChR, due to incomplete characterization of the subunit combinations that make-up the nAChR in neurons of the honey bee (or other invertebrates), which may affect both chemical binding affinity and available phosphorylation sites involved in recovery from the desensitized state (Hopfield et al., 1988; Thany et al., 2007). Although progress has been made in characterizing the composition of the nAChR subunits, most recombinant hybrid nAChRs evaluated consist of a combination of both insect and vertebrate subunits (Ihara et al., 2007). Therefore, the composition and activity of insect subunits alone have not been elucidated nor evaluated. Further, concentrations and durations of agonist exposure that would lead to a prolonged desensitized state of the receptor, effectively inactivating it, are uncertain. Research focused on characterization of insect nAChR, with evaluation of temporal and dosimetric concordance would provide greater understanding of the mechanism through which activation of the nAChR can lead to desensitization and subsequent downstream events.

3.5. KE2: mitochondrial dysfunction

Mitochondrial dysfunction is a KE directly downstream of nAChR activation. This KE is involved in four AOPs, two with altered Ca^{2+}-calmodulin activated signal transduction directly downstream (Fig. 1; AOP3 and AOP4), one with impaired hive thermoregulation (Fig. 1; AOP5), and the other with weakened colony as the immediate downstream KE (Fig. 1; AOP6). In accordance with best practices in AOP development, this KE is measureable (Table 2). Mitochondrial dysfunction occurs at the cellular level of biological organization.

3.5.1. Normal biology

Mitochondria play a critical role in cellular respiration leading to the production of adenosine triphosphate (ATP), the cell's primary energy source (Lin and Sheng, 2015). Additionally, mitochondria are integral in processes such as Ca^{2+} storage and release for cell signaling, production of heat, and mediation of cell growth and death (Hajnóczky et al., 2006). Mitochondrial dysfunction manifested by inhibition of the electron transport chain and ATP production, perturbations in Ca^{2+} signaling, interference with apoptosis or antioxidant status could lead to a number of adverse responses (e.g., Rego and Oliveira, 2003).

3.6. KER 2: nAChR activation leads to mitochondrial dysfunction

The KER linking nAChR activation to mitochondrial dysfunction is involved in four AOPs (Fig. 1; AOP3, 4, 5, and 6). However, uncertainty exists as to the presence of the nAChR in invertebrate mitochondria. Evaluation of WoE for this KER provides evidence that nAChR are present in mitochondria and that activation of the nAChR can lead to mitochondrial dysfunction.

3.6.1. Consideration of biological plausibility and empirical support

In mammals, nAChRs have been identified on the mitochondrial outer membrane, providing plausibility that perturbation of the nAChR by a chemical stressor could lead to impacts on the mitochondria (Gergalova et al., 2012; Gergalova et al., 2014; Kalashnyk et al., 2012; Lykhmus et al., 2014). Further, studies with both honey bees and bumble bees (*Bombus terrestris*) demonstrate adverse impacts on mitochondria when exposed to nAChR agonists (Moffat et al., 2015; Nicodemo et al., 2014). Specifically, mitochondria isolated from the head and thorax of honey bees and exposed to 25–100 μM imidacloprid demonstrated inhibition of the respiratory chain, specifically state-3 respiration with decreased ATP production. Moffat et al. (2015) described mitochondrial depolarization (i.e., loss of membrane potential) in bumble bee neurons upon prolonged (48 h) exposure to 1 nM imidacloprid. These cellular level impacts on the bee mitochondria upon exposure to a nAChR agonist are indicative of mitochondrial dysfunction.

In addition to the role mitochondria play in ATP production, they mediate apoptosis via Ca^{2+} accumulation through the voltage dependent anion-selective channel, activation of intra-mitochondrial protein kinases, and release of cytochrome *c* through the mitochondria permeability transition pore with further activation of caspases (Zheng et al., 2004; Green and Reed, 1998). With the identification of nAChR on the outer mitochondrial membrane in mammals, ongoing studies have

provided evidence that these receptors are coupled with the voltage dependent anion-selective channel (Gergalova et al., 2012). In mouse brain and liver, agonists of the nAChR have been shown to prevent Ca^{2+}-evoked apoptosis by interfering with intra-mitochondrial kinases (particularly phosphatidylinositol-3-kinase/Akt signaling pathway), effectively attenuating cytochrome *c* release by preventing oligomerization of voltage dependent anion-selective channel and preventing its involvement in mitochondria permeability transition pore formation (Lykhmus et al., 2014; Gergalova et al., 2014). In this case, nAChR activation leading to mitochondrial dysfunction was captured via interference with apoptotic events.

3.6.2. Weight of evidence consideration for KER2

Although biological plausibility is strong to infer that conservation of nAChR in the mitochondria exists beyond mammals, to date, studies have not evaluated the presence of the nAChR in invertebrate mitochondria. Hence, there is uncertainty in the KER due to a lack of knowledge of the presence or absence of the nAChR in insect mitochondria, resulting in determination of 'weak' WoE. Additionally, with empirical evidence suggesting that nAChR agonists prevent Ca^{2+} signaling, specifically intermitochondrial kinases, there is a potential that the KE for nAChR desensitization may be involved; however, studies are lacking. Studies to determine whether these receptors exist on mitochondria in invertebrates and their involvement (or lack of involvement) in downstream signaling would aid in clarifying this KER.

3.7. KE3: CA^{2+}-calmodulin activated signal transduction, altered

Within the AOP network, either nAChR desensitization or mitochondrial dysfunction can lead to altered Ca^{2+}-calmodulin activated signal transduction (Fig. 1; AOPs 1 to 4). This KE occurs at the cellular level of biological organization. There are several critical cell signaling processes associated with effective neuronal signaling, and perturbation at any number of points in the pathway can lead to impaired network functions (Fig. 2). However, these signaling events are rather complex to assess as discrete measurements (though several components of the pathway are measurable; Table 2); thus they are organized under the generalized term "Ca^{2+}-calmodulin activated signal transduction, altered." The sections below detail how potentially measurable impacts on a number of intracellular signaling pathways can contribute to this overall KE.

3.7.1. Normal biology (Fig. 2; steps 1–5, 8–12)

Some neuronal nAChR subunit combinations are highly permeable to Ca^{2+}, which acts as a messenger relaying extracellular information to intracellular regions and to the nucleus (Uteshev, 2012). Upon influx of Ca^{2+} into neurons via nAChR, Ca^{2+} binds to calmodulin (CaM). This complex either activates adenylyl cyclase (AC) to catalyze the conversion of ATP to 3′5′-adenosine monophosphate (cAMP), which then activates PKA, or interacts with Ca^{2+}-CaM kinase II (CaMKII) (e.g., Dajas-Bailador and Wonnacott, 2004; Sweatt, 2001). Regardless of signaling through PKA or CaMKII, both kinases activate the phosphorylation cascade via extracellular signal-related protein kinase/mitogen-activated protein kinase (ERK/MAPK), stimulating transcription of cAMP response element (CRE) binding protein (CREB) mediated genes (Impey et al., 1999). In neurons, these signaling cascades lead to the production of proteins that direct synaptic plasticity (i.e., changes in synaptic strength in response to signaling activity), which is essential to learning and memory.

3.8. KER3: nAChR desensitization leads to altered CA^{2+}-calmodulin activated signal transduction

The KER linking nAChR desensitization to altered Ca^{2+}-calmodulin activated signal transduction is involved in two AOPs (Fig. 1; AOP1 and 2). Due to a lack of empirical data describing nAChR desensitization, this relationship is primarily based on biological plausibility. As described above, Ca^{2+} signaling is essential for initiating the CREB signaling cascades (Fig. 2; step 2 Calcium influx), required for normal neuronal function; therefore, the WoE for this KER focuses on evidence demonstrating that desensitization can impact Ca^{2+} signaling and therefore downstream signaling.

3.8.1. Consideration of biological plausibility and empirical support

Empirical evidence demonstrates that desensitization of nAChRs in honey bee Kenyon cells leads to antagonistic behavior of the receptor, effectively closing the receptor (Deglise et al., 2002) as described in the KE1 section. Closure of the receptor, due to prolonged desensitization is likely to impede Ca^{2+} signaling across the nAChR and, hence, downstream components of the pathway, although studies specifically evaluating such desensitization and impacts on Ca^{2+} signaling in the insect neuron are lacking. However, Christen et al. (2016) evaluated PKA and CREB gene expression in the brain after oral exposure of honey bees to neonicotinoids. Protein kinase A transcripts were significantly decreased after 72 h of exposure to 3 ng imidacloprid/bee and after 48 h of exposure to 0.3 ng clothianidin/bee (Christen et al., 2016). Additionally CREB was significantly decreased after 48 h of exposure to 3 ng imidacloprid/bee and increased after a 24 h exposure to 0.3 ng clothianidin/bee. Although desensitization was not assessed directly, the patterns in gene expression for PKA and CREB upon imidacloprid exposure for an extended duration provide some support that a relationship exists between nAChR signaling and Ca^{2+}-calmodulin activated signal transduction.

3.8.2. Weight of evidence for KER3

Although there is evidence from mammalian studies that nAChR desensitization could lead to impacts on Ca^{2+}-calmodulin activated signal transduction (KE3) and impaired signaling downstream (e.g., Fucile, 2004; Quick and Lester, 2002; Dani and Bertrand, 2007; Marszalec et al., 2005), there are uncertainties associated with the KER. This uncertainty is related to concentrations and durations of agonist exposure that would lead to nAChR desensitization to the degree that there is an impact on downstream signaling. Such uncertainty leads to the WoE assignment of 'weak.' Focused studies on impacts of nAChR desensitization on downstream neuronal signaling in insects, particularly Ca^{2+} signaling, would be of interest to better capture the relationship between these KEs.

3.9. KER4: mitochondrial dysfunction leads to altered CA^{2+}-calmodulin activated signal transduction

The KER for mitochondrial dysfunction leading to altered Ca^{2+}-calmodulin activated signal transduction is present in two AOP descriptions (Fig. 1; AOP3 and 4). The relationship linking these events requires a WoE assessment that provides evidence that mitochondrial dysfunction affecting ATP production could lead to perturbation of the downstream CREB signaling cascade.

3.9.1. Consideration of biological plausibility and empirical support

As noted in the altered Ca^{2+}-calmodulin activated signal transduction KE description, upon activation by Ca^{2+}-CaM, ACs convert available ATP to cAMP, a critical molecule for neuronal function. It has been reported that a single neuron in the human brain can consume ~4.7 billion ATP molecules per second (Nicholls and Budd, 2000). Therefore, a continuous source of mitochondrial ATP is essential for neuronal function and synaptic plasticity (Cheng et al., 2010). As described as a component of the normal biology for KE3, the mitochondria is directly involved in supplying ATP to AC for conversion to cAMP, which acts as a second messenger involved in the signaling pathway leading to CREB/CRE activation (Fig. 2). Therefore, ATP production by the mitochondria and availability to AC is essential for proper network function in neurons.

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3.9.2. Weight of evidence for KER4

Although it is biologically plausible that mitochondrial dysfunction which impairs ATP production could lead to dysregulation of signaling in neuronal networks in bees, empirical studies are lacking and therefore cannot add evidence to this KER. Thus, the link between mitochondrial dysfunction and altered Ca^{2+}-calmodulin activated signal transduction was assigned a WoE rating of 'weak' (Table 3).

Greater knowledge of the presence (or absence) of nAChR in invertebrate mitochondria (a previously identified research gap) and additional research focused on the interplay between impacts on mitochondria and effects on the CREB pathway would be required to strengthen this KER.

3.10. KE4: learning and memory, impairment

The KE of impaired learning and memory is directly downstream of altered Ca^{2+}-calmodulin activated signal transduction and upstream of abnormal foraging activity and behavior. This KE is found in four AOPs (Fig. 1; AOP1, 2, 3, and 4), and occurs at the biological level of organization of the individual.

3.10.1. Normal biology

The prefrontal cortex and frontostriatal neuronal circuits have been identified as the primary sites of higher-order cognition in vertebrates, whereas invertebrates utilize paired mushroom bodies, shown to contain ~300,000 neurons in honey bees (Menzel, 2012; Puig et al., 2014).

Learning and memory are cognitive functions defined as the process of acquiring memory and the behavioral change caused by an experience, respectively (Okano et al., 2000). Central to these processes are changes to the brain where learning leaves physical memory traces, which change neurotransmission efficacy and communication between neurons inducing synaptic plasticity and further memory retention (Okano et al., 2000; Menzel, 2012). These alterations to neuronal network activity ultimately lead to adaptive behavior (Szyszka et al., 2008).

Learning and memory have been studied for decades. The most basic form of learning is associative learning where new associations are made between events in the environment. Classical conditioning is one form of associative learning that was made famous by Ivan Pavlov's experimentation with dogs and salivation (Pavlov, 1927). Classical conditioning protocols have been developed/used with endpoints in a wide range of species, including honey bees and bumble bees (Moore, 2002; Stanley et al., 2015; Piiroinen and Goulson, 2016).

For over 50 years an assay for evaluating olfactory conditioning of the proboscis extension reflex (PER) has been used as a reliable method for evaluating appetitive learning and memory in honey bees (Guirfa and Sandoz, 2012). These experiments pair a conditioned stimulus (e.g., an odor) with an unconditioned stimulus (e.g., sucrose) provided immediately afterward, which elicits the proboscis extension (Menzel, 2012). After conditioning, the odor alone will lead to the conditioned PER. This methodology has aided in the elucidation of five types of olfactory memory phases in honey bee, which include early short-term memory, late short-term memory, mid-term memory, early long-term memory, and late long-term memory (Guirfa and Sandoz, 2012). These phases are dependent on the type of conditioned stimulus, the intensity of the unconditioned stimulus, the number of conditioning trials, and the time between trials. Where formation of short-term memory occurs minutes after conditioning and decays within minutes, memory consolidation or stabilization of a memory trace after initial acquisition leads to mid-term memory, which lasts 1 d and is characterized by activity of the cAMP-dependent PKA (Guirfa and Sandoz, 2012). Multiple conditioning trials increase the duration of the memory after learning and coincide with increased Ca^{2+}-calmodulin-dependent PKC activity (Guirfa and Sandoz, 2012). Early long-term memory, where a conditioned response can be evoked days to weeks after conditioning requires translation of existing mRNA, whereas late long-term memory requires de novo gene transcription and can last for weeks (Guirfa and Sandoz, 2012). Knowledge gleaned from PER assays has not only increased neurobiological understanding of memory but has also provided a method for assessing the effects of chemical perturbation on learning and memory in honey bees.

3.11. KER5: altered CA^{2+}-calmodulin activated signal transduction leads to impaired learning and memory

The KER, linking altered Ca^{2+}-calmodulin activated signal transduction to impaired learning and memory, is found in four of the developed AOPs (Fig. 1; AOP1 to 4). This KER is supported by both biological plausibility and empirical data.

3.11.1. Consideration of biological plausibility and empirical support

Because the KE of altered Ca^{2+}-calmodulin activated signal transduction consists of a complex biological pathway, the WoE described below encompasses the signaling cascade initiated via Ca^{2+} movement through the nAChR to downstream events leading to impacts on proteins involved in long-term memory (Fig. 2).

3.11.2. CREB signaling pathways and memory

Neuronal health relies on Ca^{2+} homeostasis, whereby imbalanced Ca^{2+} in the cytosol can lead to neuronal damage or loss. In humans, such Ca^{2+} imbalance has been associated with neurodegenerative disorders such as dementia and Alzheimer's disease (Bezprozvanny and Mattson, 2008; Uteshev, 2012).

Invertebrate and vertebrate studies have shown that disruption of signaling pathways leading to CREB-regulated gene transcription can adversely impact long-term memory (Carlezon et al., 2005; Muller, 2000). In PC12 cells and primary neuron cultures, Ca^{2+} activates PKA, PKC, and the ERK/MAPK cascade, which are involved in CRE-mediated transcription (Impey et al., 1998). Activation of ERK/MAPK is required for the formation of contextual and spatial memories in mammals, with impairments to this signaling cascade likely contributing to cognitive decline. Analysis of the honey bee genome has revealed genes encoding PKA subunits (both regulatory and catalytic) and a gene encoding CREB, providing further evidence that this pathway occurs in bees (Eisenhardt et al., 2006). The pathway linking cAMP to CREB gene transcription may also regulate functions inside the mitochondria, as cAMP, PKA, and CREB bound to mitochondrial DNA (mtDNA) have been detected in mammals (Valsecchi et al., 2013). Further research is needed to elucidate the role of this pathway in subcellular compartments. Nonetheless, with key components of this pathway identified in both neurons and mitochondria, and knowledge that it is important for normal neurological function, it is biologically plausible that disruption of this pathway may lead to detrimental impacts on memory.

3.11.3. Adenylyl cyclase inhibition and impaired memory (Fig. 2; step 4)

In mammals ACs are regulators of the second messenger cAMP, necessary for downstream activation of cAMP-dependent protein kinases (Kamenetsky et al., 2006). In the honey bee, seven ACs have been identified, with transmembrane AmAC8 likely belonging to the Ca^{2+}-CaM activated enzyme family (Balfanz et al., 2012). Matsumoto et al. (2014) injected pharmacological inhibitors of AC directly into the brain of honey bees and observed impairment of long-term memory. Such studies with inhibitors provide evidence of the essentiality of proper AC signaling.

3.11.4. Protein kinase A and impaired memory (Fig. 2; steps 6 and 9)

Under normal biological conditions PKA is activated by cAMP. In fact, it has been demonstrated in transgenic mice expressing an inhibitory form of PKA, which reduces PKA activity in the hippocampus, that a significant reduction in late phase long-term potentiation (i.e., repetitive activation of synapse leading to a long-lasting increase in synaptic strength) and deficits in long-term memory occur (Abel et al., 1997). These adverse effects also translate to *Drosophila* sp., as flies carrying

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mutations in PKA exhibit memory deficiencies (Davis, 1996; Drain et al., 1991). Evidence also indicates that prolonged PKA activation in antennal lobes of honey bees occurs upon multiple conditioning trials, forming long-term memory (Muller, 2000). Downregulation of PKA from direct injection of anti-sense oligonucleotides to the catalytic subunit of PKA into the honey bee brain, shown to reduce PKA activity by as little as 10–15%, leads to long-term memory impairment 1 d after training (Fiala et al., 1999). Together these data suggest the importance of neuronal PKA activity in memory formation.

3.11.5. CaMKII and impaired memory (Fig. 2; step 8)

Calcium-CaM kinase II has been well studied relative to its action in Ca^{2+}-dependent synaptic changes. This kinase is made up of two catalytic subunits, α and β, which remain inactive in their resting state via interaction with an auto-inhibitory domain (Mochly-Rosen, 1995). Binding of Ca^{2+}-CaM to the autoinhibitory domain activates the kinase, leading to autophosphorylation and transition to its Ca^{2+}-independent form. When CaMKII assumes its Ca^{2+}-independent state, the protein can remain active even when Ca^{2+} levels decline (Mochly-Rosen, 1995). In mammals CaMKII is abundant in the hippocampus, a region of the brain important for memory formulation (Lisman et al., 2002). It has been demonstrated that reduced CaMKII function, via mutation or pharmacological inhibition, leads to impaired learning and long term potentiation in mice (Malinow et al., 1989; Scholl et al., 2015). *Drosophila* also shows impaired learning and memory following inhibition of CaMKII (Scholl et al., 2015). Knockdown with RNA interference and pharmacological inhibition of CaMKII in honey bee was shown to impair both early and late- long-term memory (Scholl et al., 2015). Hence, empirical evidence indicates that disruption of CaMKII leads to impaired memory across various vertebrate and invertebrate models.

3.11.6. Inhibition of MAPK cascade and impaired memory (Fig. 2; step 10)

The MAPK cascade consists of three kinases: MAP kinase kinase kinase (MEKK), which activates MAP kinase kinase, subsequently activating MAPK (Sharma and Carew, 2004). Upon activation, MAPKs phosphorylate transcription factors, including CREB. Activation of extracellular signal-regulated protein kinases (ERK1 and ERK2), members of the MAPK family, have been implicated as key regulators of synaptic plasticity (Thomas and Huganir, 2004). The earliest work demonstrating the essentiality of these kinases in plasticity used a pharmacological MEK inhibitor, essentially blocking long-term potentiation in rat hippocampal slice preparations (English and Sweatt, 1997). Water maze training trials in rodents demonstrated that MEK inhibition led to impaired memory retention but did not impact memory acquisition (Hebert and Dash, 2002; Selcher et al., 1999).

Empirical evidence supports the dependency of ERK translocation on PKA activation, as inhibition of PKA leads to reduced ERK localization in the nucleus of PC12 and hippocampal neurons (Impey et al., 1998; Yao et al., 1998). Graves et al. (1993) provided evidence that ERK does not directly interact with PKA; however, the enzyme may indirectly modulate either dimerization or mechanisms of translocation of ERK into the nucleus. Such studies demonstrate the coordinated signaling necessary for proper neuronal function.

Other studies have demonstrated that abnormal activation of nAChR and downstream disruption of ERK/MAPK lead to impaired cognition. For example, beta amyloid (Ab_{1-42}) peptide, which accumulates in the brains of patients with Alzheimer's disease, binds specifically and potently to α7-nAChRs, functionally blocking the current responses evoked by activation of α7-nAChR (Dajas-Bailador and Wonnacott, 2004). Chronic exposure of rat hippocampal slices to Ab_{1-42} increases the level of α7-nAChRs, which correlates with down-regulation of ERK/MAPK and phosphorylated CREB. Animals with elevated α7-nAChR levels show learning and memory deficits, reflective of defective ERK/MAPK and CREB signaling, caused by Ab_{1-42} binding and subsequent desensitization of α7-nAChRs.

3.11.7. Inhibition of CREB transcriptional activation leads to impaired memory (Fig. 2; steps 11–12)

Research in both vertebrates and invertebrates indicates that long-term memory requires CREB-dependent transcriptional activation and protein synthesis (Kandel, 2012; Silva et al., 1998). The CREB is a phosphorylation-dependent nuclear transcription factor responsible for recognition and activation of genes with the conserved cAMP response element (CRE) in their promoters (Silva et al., 1998). Further, CREB is activated via phosphorylation of key serine residues by a variety of kinases including PKA, CaMKII, or ERK/MAPK (Kandel, 2012). Phosphorylated CREB then binds its coactivator, CREB-binding protein (CBP), leading to unwinding of the DNA and transcription of genes important for long-term memory. The cAMP response element binding protein 2 (CREB2) can repress CREB1 transcription; thus, in addition to CREB1 activation, CREB2 repression must be removed for long-term synaptic potentiation (Martin et al., 1997). Protein kinase A has been shown to directly phosphorylate CREB1 but not CREB2, which lacks a PKA phosphorylation site and contains conserved consensus sites for phosphorylation by MAPK (Martin et al., 1997). Therefore, MAPK facilitates the activation of CREB1-mediated transcription. It has been demonstrated in California sea hare (*Aplysia californica*) sensory neurons that CREB1 leads to the expression of several immediate-response genes that stabilize short-term facilitation (i.e., repetitive activation of synapse leading to a short-lived increase in synaptic strength), as well as other transcription factors that activate genes essential for new synaptic connections and are critical for long-term potentiation (Kandel, 2012).

Although there is strong biological plausibility that disruption of the indicated pathways leads to CREB activation of CRE-mediated genes to impair memory, and that these pathways are conserved in vertebrates and invertebrates alike, there are substantial research gaps that must be addressed to understand whether desensitization of the nAChR in honey bee can adversely impact these signaling pathways.

3.12. KER 6: nAChR activation leads to impaired learning and memory

The KER for nAChR activation leading to impaired learning and memory is present in four AOP descriptions (Fig. 1; AOP1-4). Typically studies with bees utilize a nAChR agonist, and assess learning and memory with the PER assay, a few of which are described below.

3.12.1. Consideration of biological plausibility and empirical support

Knockdown of the α8-nAChR in honey bee mushroom bodies impairs memory retrieval at 24 and 48 h post PER conditioning (Louis et al., 2012). Yang et al. (2012) provided evidence that exposure of bee larvae to 0.04 μg/L imidacloprid solution added directly to the brood cell resulted in impaired olfactory associative behavior, as measured by PER, in adult bees. Exposure of adult bees to imidacloprid has also been reported to impair memory utilizing the PER procedure. For example, exposure to 12 ng imidacloprid/bee was shown to impair medium-term memory retention measured at 15 min or 1 h conditioning-treatment time intervals (Decourtye et al., 2004). In another study, evidence for clear impacts on learning and memory were observed upon exposure of honey bees to dietary imidacloprid resulting in a dose of ~1.2 ng/bee (estimates based on consumption rates) using the PER assay (Williamson and Wright, 2013). Similarly, honey bees that were fed sucrose solution containing 10 nM imidacloprid or 1 nM thiamethoxam exhibited impaired olfactory learning and short-term memory, as evaluated using the PER (Wright et al., 2015).

3.12.2. Weight of evidence consideration for KER6

There is contradictory in vivo and in vitro evidence for the biological plausibility supporting the KER linking nAChR activation to impaired learning and memory, where moderate activation of the nAChR with an agonist leads to neuroprotection and enhanced cognition, as opposed to impaired learning and memory (Uteshev, 2012). Such inconsistency with the proposed AOP leading to impacts on learning and memory

may be due to differences in test chemical dose and time course of exposure, desensitization state of the receptor, and/or species differences in receptor subunit combinations and toxicological profiles (Uteshev, 2012). Thus, while the association between nAChR activation and impaired learning and memory is relevant and supported by the literature, improved quantitative understanding of the conditions modulating effects (in terms of severity and duration of the perturbation) and the "biological tipping points" at which neuroprotective mechanisms are overwhelmed or exhausted is required to understand whether exposures would be sufficient to elicit impacts on learning and memory as opposed to neuroprotection and enhanced cognition. Overall, this KER was assigned a WoE call of 'moderate.'

3.13. KE5: foraging activity and behavior, abnormal

Directly downstream from impaired learning and memory is the KE of abnormal foraging activity and behavior. This KE is present in four of the developed AOPs (Fig. 1; AOP 1 to 4) and can be either directly upstream of the KE weakened colony, or of KE abnormal role change within caste. Tracking devices have been identified as useful for measuring this KE (Table 2), which occurs at the individual and colony levels of biological organization.

3.13.1. Normal biology

As eusocial insects, honey bees rely on the worker bee caste to forage for nectar, pollen, and water. Foraged water can be used for evaporative cooling of the hive during warm weather (as reviewed by Jones and Oldroyd, 2006). Nectar and pollen collected by the foragers are the sole food source for the colony, with nectar providing carbohydrates and pollen providing lipids, protein, vitamins, and essential minerals (Brodschneider and Crailsheim, 2010). Upon returning to the hive, forager bees identify non-foraging, food-storing hive bees and deliver their collection by regurgitating nectar carried back in their honey stomach (i.e., foregut of proventriculus; Free, 1959). The hive bees place the nectar in wax cells for processing into honey. Hive bees also aid foragers in unloading pollen from the pollen baskets (corbicula) on the forager's hind legs and place it in cells where it is mixed with nectar to form bee bread, which is stored for consumption by the colony (Winston, 1987). Foragers consume only small amounts of the food they collect. Hive bees consume the food they receive in order to produce protein-rich royal jelly and brood food, which they use to nourish both the queen and the developing brood (Winston, 1987). During winter, the colony survives on the pollen and nectar that was stored as bee bread and honey over the spring, summer, and fall seasons (Seeley and Visscher, 1985).

The act of foraging is a perilous and metabolically challenging task that is typically carried out by worker bees in the later stages of life (Woyciechowski and Moroń, 2009). However, the timing of the role change from hive bee to forager can vary depending on the needs of the colony. There are environmental, hormonal, and social cues that determine when and how often foragers search for food and fluid, including weather, abundance or scarcity of food resources, magnitude of food stockpiled in the hive, health of the colony, and size of the brood (Dreller and Tarpy, 2000). Such cues initiate physiological changes involved in the transition of a worker bee to foraging, which include changes to flight muscles and metabolic rate. These changes accommodate the reported 70-fold increase in oxygen consumption needed to sustain physical and cognitive activities of the forager bee (Kammer and Heinrich, 1978). It has been documented that the volume of neuropil in mushroom bodies is increased by approximately 15%, and the somata of the Kenyon cells decreased by approximately 29% in foragers compared to day-old bees (Withers et al., 1995). Change in lipid stores also occurs in forager bees prior to foraging, whereby their abdominal lipid is reduced to approximately half that of nurse bees (Chang et al., 2015; Toth and Robinson, 2005). Further, there is low protein content in the forager's fat body cells, and vitellogenin (Vtg; egg yolk) protein production is significantly reduced, while juvenile hormone levels are significantly increased (Toth and Robinson, 2005). Another change which occurs at the stage where worker bees become foragers is that their flight muscle fiber thickness decreases and diameter of the myofibrils, which contain the contractile filaments, increases in preparation for prolonged flight during foraging (Correa-Fernandez and Cruz-Landim, 2010).

3.14. KER7: impaired learning and memory leads to abnormal foraging activity and behavior

The KER for impaired learning and memory leading to abnormal foraging is present in four AOP descriptions (Fig. 1; AOP1 to 4). This relationship has been well studied.

3.14.1. Consideration of biological plausibility and empirical support

Foraging involves multiple complex cognitive processes, including communication between colony members, learning and memory retention of navigational cues, flight path decisions, information processing, resource selection and food collection, predator avoidance, and homing. In preparation for foraging, a new forager embarks on a number of orientation flights to identify and learn landmarks for navigation to and from the hive (Capaldi et al., 2000). Such navigational information includes using the sun azimuth as a visual compass and for estimating distance (Fischer et al., 2014). Successful foragers and scouts will recruit nest mates to profitable food collection locations using an intricate behavior termed the waggle dance. The dance is used to communicate direction, distance, and quality of the food source to other foragers. Foragers attending the dance then process the communication to locate beneficial foraging sites to maximize efficiency of foraging efforts. These interactions require learning from previous experiences and acquiring memories to both display the waggle dance to nest mates and utilize information from the dance.

Given the complexity of honey bee foraging activities, it is biologically plausible that any perturbation to cognitive function, whether it impairs navigation or communication capabilities, could ultimately lead to abnormal foraging behavior and activity.

Several studies have reported varying effects of neonicotinoids on cognitive functions in honey bees and foraging behavior at a range of exposure levels. In order to establish a link between learning and memory and chemical impacts on foraging, studies typically train bees to feed from an artificial feeder (with or without a conditioned stimulus, typically an odor) and ensure bees return to a specific feeding site (pre-exposure), expose the bees to a test chemical, and then evaluate changes in parameters associated with foraging success (post-exposure). For example, honey bees orally treated with 1.3 ng thiacloprid/bee demonstrated significantly delayed initiation of flight back to the hive from specified forage sites compared to initiation of return flights pre-exposure (Fischer et al., 2014). Additionally, those treated with either 11.3 ng imidacloprid/bee or 1.3 ng thiacloprid/bee showed a significant increase in failure to return home and exhibited directional changes in the flight vector leading back to the hive, while exposure to 2.5 ng clothianidin/bee led to significantly longer total flight paths during the homing phase (Fischer et al., 2014).

In another study, where honey bees were trained to feed on an artificial feeder, exposed to the test chemical, captured, and then released to locate the feeder, it was observed that imidacloprid treatment increased the time it took for the bees to leave the release site, seemingly in a dose dependent manner (Bortolotti et al., 2003). Further, abnormal flying behavior, which the authors attributed to disorientation, was reported and an inability to return to the hive was noted at higher test concentrations (Bortolotti et al., 2003). Honey bees exposed to 1 ng thiamethoxam/bee (via sucrose solution) experienced significantly higher mortality relative to control foragers due to homing failure, as measured by radio-frequency identification tag tracking of confirmed

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foragers collected from their hive and released at locations away from the colony (Henry et al., 2012).

Another study using radar tracking of honey bee flights following exposure to several different neonicotinoids (2.5 ng clothianidin/bee, or 11.25 μg imidacloprid/bee or 1.25 μg thiacloprid/bee) also reported adverse effects on homing, where the rate of bees returning to the hive was significantly lower than controls (Fischer et al., 2014). Visitation intervals to an artificial feeding site were also delayed when honey bees were fed a one-time dose of 50 μg imidacloprid/L sucrose solution (~1.82–4.33 ng/bee based on consumption data) (Yang et al., 2008). Further, dietary exposure to concentrations of imidacloprid ranging from 600 to 3000 μg/L led to a dose-dependent increase of missing bees during the following 24-h period (34.4–96.9% missing bees) (Yang et al., 2008). After the 24-h period, feeding recovery was observed in bees that had originally gone missing after exposure to concentrations <1600 μg imidacloprid/L and subsequently returned. However, those that did return did not regularly visit the feeder (Yang et al., 2008).

Using radio-frequency identification tagging, Schneider et al. (2012), described both reductions in foraging activity as well as increased foraging durations when bees were exposed to 0.5 ng clothianidin and 1.5 ng imidacloprid/bee. Tan et al. (2014) studied the ability of the Asian honey bee (*A. cerana*) to avoid the common predator hornet (*Vespa velutina*) following dietary exposure to 40 μg imidacloprid/L (~0.52 ng/bee), concluding that a significantly greater number of treated bees (1.8 fold more) put themselves at risk compared to control organisms. The study also identified impacts on foraging efficiency, with bees exposed to 20 μg imidacloprid/L or 40 μg imidacloprid/L collecting 46% to 63% less nectar, respectively, than controls (Tan et al., 2014).

In another study, upon exposure to 48 μg imidacloprid/L, honey bees demonstrated reduced food consumption and a significant decrease in foraging activity as measured by the number of trips to an artificial feeder (Ramirez-Romero et al., 2005). Bumble bees exposed for a prolonged period (28 d) to 10 μg imidacloprid/L in the diet exhibited significant impairment in their foraging performance compared with controls (Gill and Raine, 2014). In that study the imidacloprid-exposed bumble bees had significantly greater numbers of foragers throughout the duration of the experiment; however, their pollen foraging efficiency was decreased as evidenced by smaller pollen loads brought back by foragers from exposed colonies in comparison to control colonies (Gill and Raine, 2014). Further, as the foragers aged and gained experience foraging, control bees returned with larger pollen loads as expected whereas imidacloprid-exposed bees brought back smaller pollen loads regardless of age or experience (Gill and Raine, 2014).

Exposure to imidacloprid and thiamethoxam has also been shown to increase the honey bee sucrose response threshold (lowest sucrose percentage that elicits PER), as an indicator of decreased appetitive motivation and selective foraging for sweeter food sources, at doses of 0.21–2.16 ng imidacloprid/bee and 1 ng thiamethoxam/bee (Aliouane et al., 2009; Eiri and Nieh, 2012). Preference for sweeter nectar sources leads to reduced or more selective foraging by bees and is causally linked to division of labor of foraging (i.e., water, nectar, or pollen foraging) (Pankiw and Page, 2000). Therefore, studies reporting increased sucrose response thresholds and decreased appetitive motivation indicate that the colony food supply would be negatively impacted due to the lower quality of nectar collected and the greater effort needed to process that nectar into stored honey (Drezner-Levy et al., 2009; Eiri and Nieh, 2012). Finally, the waggle dance, which requires learning and memory as well as coordination, was shown to be impacted by exposure to 0.21 ng imidacloprid/bee, with treated animals performing between 4.5 and 10.5-fold fewer dance circuits compared to controls (Eiri and Nieh, 2012).

Although numerous studies report effects of neonicotinoids on foraging behavior in bees, some contradictory evidence following exposure to the neonicotinoids also has been reported (Dively et al., 2015; Franklin et al., 2004; Pilling et al., 2013). Some of these studies examined the effects on foraging after exposure to seed-treated crops; however, Dively et al. (2015) fed 10 replicate colonies 0, 2, 20, or 100 μg/kg imidacloprid over a 12-week period, reporting no adverse effects on foraging activity as measured by the number of foragers returning to the hive or the number of animals loaded with pollen pellets. Nevertheless, this study noted a 12% decrease in foraging activity compared to control during late August and early September, observations collected after the pesticide exposure period (Dively et al., 2015).

3.15. KE6: role change within caste, abnormal

The KE of abnormal role change within caste is present in two of the developed AOPs (Fig. 1; AOP2 and 4). Abnormal foraging activity and behavior is the KE directly upstream, and weakened colony directly downstream. Abnormal role change within caste occurs at the individual level of biological organization.

3.15.1. Normal biology

Like most eusocial insects, honey bees exhibit age-based division of labor and progress from nurse to forager as they age (Seeley, 1982). This type of age-related behavioral change termed age polyethism, is a genomically, nutritionally, and hormonally controlled process (Ament et al., 2010; Cheng et al., 2015). Such behavior changes in adult worker bees occur in a predictable sequence as they move from centrally located in-hive activities including cleaning brood cells (0–5 d old), to feeding brood, capping brood, trimming cappings, and attending the queen (2–11 d old), to peripherally located in-hive activities, such as grooming nest-mates, feeding nest-mates, ventilating the hive, producing wax and shaping comb cells, receiving and storing nectar, packing pollen, and processing nectar into honey and pollen into bee bread (11–20 d old), to outside activities, including guarding the hive and foraging (20+ d old) (Seeley, 1982). However, honey bees exhibit phenotypic plasticity whereby the rate of behavioral change is highly flexible, meaning that under different scenarios, based on colony needs, bees will accelerate or reverse their behavioral development. For example, to compensate for a loss of foragers, disease, or nutritional stress, bees will initiate precocious (early behavioral development) foraging (Cheng et al., 2015; Huang and Robinson, 1996). It is biologically plausible that early initiation of foraging could lead to a shortage of hive bees needed to tend to the brood, which could hinder development of the brood. In addition, precocious foraging is correlated with shorter lifespans. Therefore, bees that forage earlier tend to do so at the expense of their longevity which could impact overall colony resource acquisition and productivity (Woyciechowski and Moroń, 2009). However, the relationship may be complex given that with seasonal variation, food availability, predation pressures, and adverse weather conditions that promote greater in-hive activity, older foragers can reverse their behavior, regenerate hypopharyngeal glands, and assume roles within the hive (Huang and Robinson, 1996).

Behavioral plasticity is driven, in part, by juvenile hormone (JH) and its interplay with Vtg, acting together in a feed-back loop to control the onset of labor tasks, such as foraging (Page et al., 2012). For example, high Vtg levels suppress JH, delaying onset of foraging behavior, whereas high JH suppresses Vtg, causing a decrease in nursing behavior (Page et al., 2012). Studies exploring drivers of precocious foraging, using both treatment with a JH analog and social manipulation of a single-cohort colony of 1 d old bees in the absence of older foragers, induced precocious foraging, demonstrating that both hormonal and social interactions play a role (Chang et al., 2015; Perry et al., 2015). Active foragers produce a pheromone, ethyl oleate, which is transferred to the hive bees during trophallaxis or oral food exchange, delaying the rate at which bees transition to foraging. Therefore, if the number of foragers diminishes, recruitment to foraging can be accelerated. Additionally, allatectomy (removal of the corpora allata glands that produce JH) led to the discovery that JH is involved in modulating the speed at which bees develop into foragers, but not in activation of foraging itself (Sullivan et al., 2003). However, studies using ribonucleic acid

interference (RNAi) to knockdown Vtg production have found the protein to have a prominent role in the initiation of honey bee foraging, causing an increase in JH titer and extreme precocious foraging (3 d old bees) (Guidugli et al., 2005; Marco Antonio et al., 2008).

Vitellogenin is synthesized in fat body cells, released to the hemolymph (circulation), and taken up in developing oocytes (Corona et al., 2007). Mature honey bee queens, which lay ~1000 eggs/day, continuously synthesize Vtg at high levels, including during periods when egg laying ceases (Seehuus et al., 2006; Corona et al., 2007). However, in sterile worker bees, Vtg levels have been shown to change throughout their lives, with the highest levels observed in the long-lived winter bees and lowest in the short-lived summer foragers (Münch et al., 2015). In addition to the role Vtg plays as an egg yolk protein, it has a role in oxidative stress resistance (Corona et al., 2007; Seehuus et al., 2006; Amdam et al., 2004).

3.16. KER8: abnormal foraging activity and behavior leads to abnormal role change within caste

The KER describing abnormal foraging activity and behavior leading to abnormal role change within caste is present in two AOP descriptions (Fig. 1; AOP2 and 4). The WoE evaluation should provide evidence linking upstream and downstream events; however, evidence for this KER primarily is supported by biological plausibility.

3.16.1. Consideration of biological plausibility and empirical support

Stressors that impact foraging activity and behavior that lead to low colony food stores or loss of foragers will induce behavioral role changes within the worker caste, causing precocious foraging by young hive bees (Schulz et al., 1998; Cheng et al., 2015; Huang and Robinson, 1996). Consequences of precocious foraging may include shorter worker lifespans, less efficient foraging due to maladapted flight muscles, and higher mortality due to inexperience of young foragers that may lead to weaker colonies (Perry et al., 2015; Vance et al., 2009). However, in addition to early onset of foraging, honey bee colonies have a repertoire of compensatory responses to overcome foraging deficiencies. These responses include increasing the activity of the existing (or remaining) foraging force, cannibalizing brood, and capping brood cells early (Fewell and Winston, 1992; Khoury et al., 2013; Schmickl and Crailsheim, 2001). These latter two adaptations serve to reduce the nutritional demands of the colony.

3.16.2. Weight of evidence for KER8

It is difficult to determine when or how one or more of the compensatory mechanism(s) used by honey bees may be chosen over the others and at what point, based on amount of food present in the colony or number of forager bee losses, does the colony reach a threshold where precocious foraging is the primary solution for survival. Colony population dynamics modeling has provided some valuable insights in this regard (Khoury et al., 2011; Perry et al., 2015; Bryden et al., 2013), but corresponding field studies testing model predictions are lacking. Therefore, although there is both strong biological plausibility and supporting empirical evidence for the KER linking abnormal foraging activity and behavior to abnormal role change within the caste, the WoE for the KER was designated 'moderate' due to the uncertainties associated with how survival strategies are selected and employed by the colony. Such relationships between compensatory strategies and the conditions (quantity of food, brood size, and number of foragers) that drive initiation of precocious foraging could be with the subject of future studies.

Due to the lack of knowledge as to when precocious foraging is initiated relative to other compensatory mechanisms, and uncertainty as to when this caste shift results in an adverse as opposed to a beneficial outcome for the colony, essentiality of the KE of abnormal role change within caste cannot be determined. Typically, it has been recommended that essentiality be established to include a KE in an AOP. However, in the context of an AOP network, where multiple stressors can impact a pathway, it is more likely to identify KEs that in and of themselves may not be essential, but nonetheless are important events to capture. For example, precocious foraging in isolation could lead to positive impacts on the colony food source, but if another stressor, such as the presence of a honey bee predator, comes into play, the poor flight and predator avoidance capabilities of the precocious forager may lead to greater losses of the foraging force, enhancing the likelihood of weakening the colony.

3.17. KE7: hive thermoregulation, impaired

Impaired hive thermoregulation is a KE found in one of the developed AOPs (Fig. 1; AOP5). The KE directly upstream is mitochondrial dysfunction and the KE directly downstream is weakened colony. This KE occurs at the colony level of biological organization.

3.17.1. Normal biology

Honey bees and some bumble bees are exposed to and survive temperature fluctuations by maintaining a steady-state internal hive temperature, when brood is present, within the range of 34–36 °C and can survive even at lower in-hive temperatures of around 21 °C during winter and times of broodlessness (Fahrenholz et al., 1989; Simpson, 1961). To endure both cold and warm environments, the hive practices social homeostasis. A critical mass of bees in the colony, adequate pollen storage, and behavioral interactions ensure survival of the hive during varying weather conditions by allowing for hive thermoregulation (Jones et al., 2004).

In warm weather, hive bees cool the colony by fanning their wings and orienting themselves near the nest entrance for increased air exchange (Kronenberg and Heller, 1982). Additionally, foragers bring water to the hive for evaporative cooling and prevention of larval desiccation (Lindauer, 1954). If such measures are insufficient a number of bees will evacuate the hive and cluster outside, leaving an essential number of bees actively cooling the hive to preserve the brood within (Southwick and Heldmaier, 1987).

During winter, particularly in cooler environments, bee colonies form a winter cluster, crowding tightly together and insulating the queen and breeding cavity (Simpson, 1961). This colony coordinated clustering eliminates heat loss, prevents individual bees from experiencing chill coma (bees with thorax temperature below 9–11 °C cannot activate flight muscles for heat generation), and ensures access to food storage with minimal energy expenditure (reviewed in Jones and Oldroyd, 2006). Within the clusters, worker honey bees provide endothermic heat through shivering thoracic flight muscles as a primary mechanism for maintaining thermal homeostasis in the brood cells and throughout the colony (Stabentheiner et al., 2003).

An additional consideration is that thermoregulatory impairment may become more likely as extreme weather events cause temperatures to fluctuate more rapidly, reaching stress-inducing conditions. In general, however, studies evaluating hive thermoregulation following exposure to chemical and/or non-chemical stressors are lacking.

3.18. KER9: mitochondrial dysfunction leads to impaired hive thermoregulation

The KER for mitochondrial dysfunction leading to impaired hive thermoregulation is present in one AOP description (Fig. 1; AOP5). This KER is supported by the biological plausibility that mitochondrial ATP is necessary as energy to support flight muscle contractions that facilitate hive thermogenesis.

3.18.1. Consideration of biological plausibility and empirical support

The number of mitochondria in a cell is dependent on the metabolic needs of that cell, where greater numbers are required for tissues with

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high energy demand. In bees, the greatest numbers of mitochondria are found in the nervous system, heart, and thorax (Nicodemo et al., 2014). The flight muscles on the thoraces of flying insects depend heavily on ATP as cellular energy, as insect flight has been recognized as one of the most energetically demanding processes of locomotion (Martinez-Cruz et al., 2012). Further, the flight muscles are vital for shivering thermogenesis. In fact, shivering thermogenesis has been reported to increase the metabolic rate in bees by a factor of 25 (Southwick and Heldmaier, 1987).

Recent studies evaluating the impact of neonicotinoids on both honey bee and bumble bee mitochondria report evidence of dysfunction (Moffat et al., 2015; Nicodemo et al., 2014). Specifically, mitochondria collected from honey bee thorax and head were isolated and incubated with 25 μM–100 μM imidacloprid in the presence of respiratory substrates, which activate various complexes of the electron transport chain, and oxygen consumption and ATP production were measured after a 15 min exposure (Nicodemo et al., 2014). State-3 respiration (defined as ADP-stimulated respiration) was significantly inhibited at concentrations ≥50 μM in mitochondria from both tissues, indicating a direct effect on the electron transport chain with significant inhibition of ATP synthesis (Nicodemo et al., 2014). Additionally, a 2-d exposure to imidacloprid concentrations at 1 nM (in diet) in bumblebees led to mitochondrial depolarization (Moffat et al., 2015). Overall, it is biologically plausible to suggest that mitochondrial dysfunction, which leads to decreased ATP production, would have adverse impacts on energy-demanding activities involving flight muscles and neuronal signaling in the honey bee.

Given the mechanisms bees employ for thermoregulation, it is plausible that decreases in the number of worker bees available to form the overwinter cluster; impairment of shivering thermogenesis and fanning behaviors necessary for heating and cooling, respectively; or insufficient honey or pollen storage (via abnormal foraging activity and behavior) to sustain the colony through cold weather can result in impaired thermoregulation, brood mortality, and colony death.

3.18.2. Weight of evidence consideration for KER9

Although the proposed relationship between mitochondrial dysfunction and impaired hive thermoregulation appears highly plausible, there is a lack of empirical evidence directly exploring this relationship (Martinez-Cruz et al., 2012; Southwick and Heldmaier, 1987). Therefore, the WoE for the KER was designated as 'weak.' While studies have demonstrated that exposure to neonicotinoids adversely affect mitochondrial function in bees (Moffat et al., 2015; Nicodemo et al., 2014), and that neonicotinoids impact honey bee thermoregulation as measured by thorax temperature (Tosi et al., 2016), in future work it would be desirable to link these effects.

3.19. KE8: colony weakened (Fig. 3)

The KE of weakened colony is a component of all the developed AOPs (Fig. 1). It immediately precedes the AO of colony death, and is directly downstream of four different KEs, including abnormal foraging activity and behavior, abnormal role change within caste, impaired hive thermoregulation, and mitochondrial dysfunction. This KE is at the colony level of biological organization.

3.19.1. Normal biology

Both Oregon and Washington Departments of Agriculture (in the U.S.) have developed regulations to mandate minimum standards for colony strength and quality to ensure that rented (managed) honey bee colonies will provide sufficient commercial pollination services for dependent crops provided by honey bees (Sagili and Burgett, 2011). Although these regulations are typically not enforced, they serve as useful guidance for assessing colony health. The characteristics evaluated to determine the strength/health of honey bee colonies, include adequate numbers of adult bees, presence of sealed and open brood, adequate amounts of stored pollen, nectar and sealed honey, the absence of pests and disease, and the presence of a queen that lays eggs in consistent and tight patterns, with limited eggless cells (Sagili and Burgett, 2011). If the colony is weakened by any one (or a combination) of these factors for an extended period, a critical point can be reached that will lead to colony failure. Through honey bee population dynamics models, it has been demonstrated that loss of foragers leading to precocious foraging of young bees may restore the overall foraging capacity, but the brood rearing capacity of the colony might be reduced (Khoury et al., 2011). Further, as noted above, precocious foragers are less effective and resilient, causing the forager death rate to increase. The model predicts that sustained forager losses that reduce the force by two-thirds would place a colony at risk for failure (Khoury et al., 2011). Additionally, proper brood rearing is essential to the

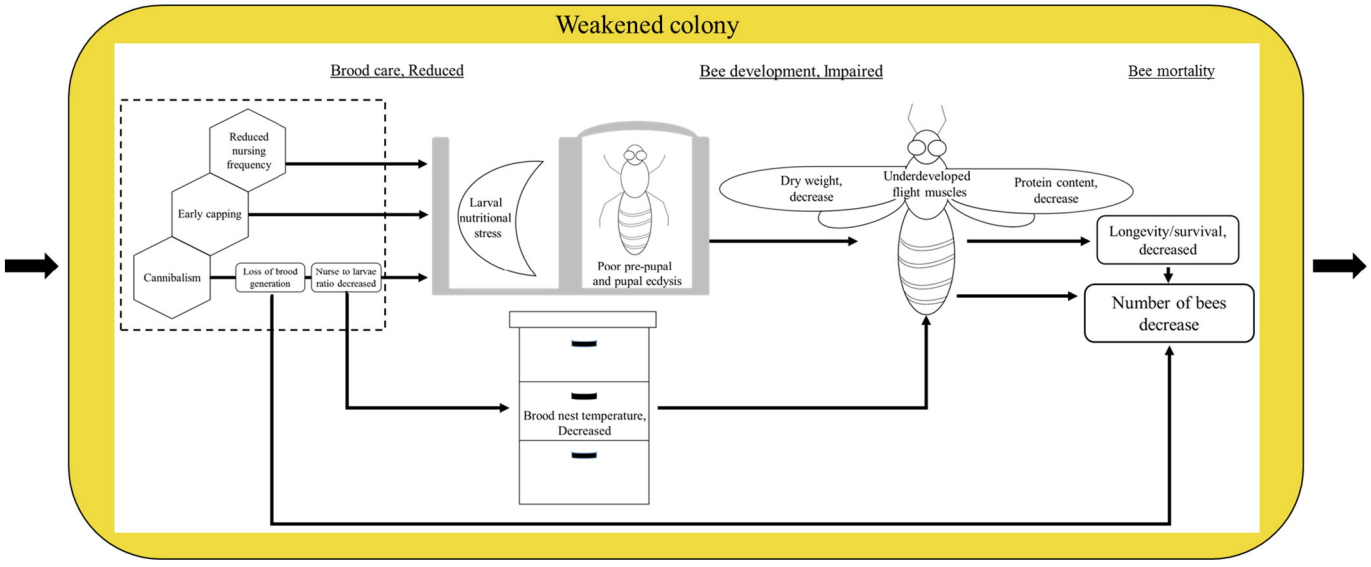

Fig. 3. Figure depicting the key event of weakened colony (KE8 in Fig. 1). A colony can be considered weakened following perturbations that lead to reduced brood care, impaired development, or mortality. The dashed box represents those events that are typically employed as compensation methods to prevent weakened colony, though in and of themselves can also adversely impact the colony. The upstream arrow can be preceded by abnormal foraging activity and behavior, abnormal role change within caste, impaired hive thermoregulation, or mitochondrial dysfunction.

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development of healthy adult bees; thus, a precocious shift in the worker caste from brood care to foraging may leave the brood vulnerable to a lack of care and inadequate sustenance.

3.20. KER10: mitochondrial dysfunction leads to weakened colony

The KER linking mitochondrial dysfunction to weakened colony is present in one AOP (Fig. 1; AOP6). Available literature has not explicitly evaluated this linkage in honey bee colonies; thus, the primary WoE supporting this linkage comes from biological plausibility.

3.20.1. Consideration of biological plausibility and empirical support

Mitochondria generate endogenous reactive oxygen species as byproducts of oxidative phosphorylation. For example, 0.4–4% of all oxygen consumed during this process can be converted to the superoxide radical (Melov, 2000). Since mtDNA lacks protective histones, it is particularly susceptible to reactive oxygen species. However, in functioning mitochondria, antioxidant activities and repair mechanisms defend against damage to mtDNA (Boesch et al., 2011). In instances where these damage prevention or repair mechanisms are overwhelmed or functioning inadequately, such as during mitochondrial dysfunction, mtDNA mutations can be introduced or apoptosis induced. Mutations to mtDNA have been associated with aging in mammals and model invertebrates such as nematodes and fruit flies (Bishop and Yankner, 2010). Inheritance of the mitochondrial genome is different from nuclear genomes in that transmission to offspring is strictly uniparental; in honey bee, mtDNA is maternal in origin (Meusel and Moritz, 1993). Therefore, unrepaired mtDNA mutations have the potential to be transgenerational. Downregulation of genes important for maintenance of mtDNA has been reported in forager bees compared to other castes, reducing mitochondrial translation and translation precision (Seehuus et al., 2013). Therefore, it may be that castes that are predisposed to higher mtDNA mutational loads would be particularly susceptible to the aging effects brought about by mitochondrial dysfunction. An example supporting the biological plausibility of a relationship between mtDNA integrity and aging can be seen with mtDNA-mutated mice that accumulate progressive and random point mutations with reduced proofreading capabilities, which culminate in premature aging and reduced lifespan (Lagouge and Larsson, 2013). Accordingly, more studies are warranted to better understand how mitochondrial dysfunction may impact aging in bees and how these lifespan changes would ultimately weaken the colony.

3.21. KER11: abnormal foraging activity and behavior leads to weakened colony

The KER linking abnormal foraging activity and behavior to weakened colony is found in two AOP descriptions (Fig. 1; AOP 1 and 3). Abnormal foraging can ultimately lead to poor food supply and/or bee death. The biological plausibility of the linkage between poor food supply and poor colony strength provides the primary WoE for this relationship.

3.21.1. Consideration of biological plausibility and empirical support

As described in the previous section (KE5: FORAGING ACTIVITY AND BEHAVIOR, ABNORMAL), the honey bee colony acquires resources from foraging outside the hive and returning with pollen and nectar, where it is processed, stored, and consumed. In addition to food and water, propolis is collected by bees from trees. Propolis is a resinous mixture that has antibacterial and antifungal properties and is used to seal, envelop, and protect hive cavities (reviewed by Ghisalberti, 1979). Decreases in resource influx or loss of worker bees due to abnormal foraging activity and behavior can lead to detrimental effects on the colony such as reduced brood care, nutritional stress, impaired larval development, increased susceptibility to pathogens, and bee mortality (Fig. 3).

The early stages of honey bee development consist of a pre-capping period and a post-capping period; the former has an egg and feeding-larval stage, and the latter includes a spinning larval stage, a prepupal stage, and an imaginal stage, prior to emergence of the adult (eclosion) from the capped cell (Jay, 1963). The feeding larval stage of honey bee development, which takes place after eclosion, consists of 5.5 days of constant feeding by nurse bees, accompanied by rapid growth of the larva and multiple molts (Fyg, 1959). Worker larvae reach average weights of 144 to 162 mg prior to the pre-pupal and cell-capping stage, and after metamorphosis upon emergence from their cells have average weights of 81 to 120 mg as young adults (reviewed by Jay, 1963). Proper nutrition, specifically the amount and quality provided by the nurse bees during the larval stage, directly affects the weight and size of larvae and is therefore considered a critical factor for normal development (Brodschneider and Crailsheim, 2010; US EPA, 2016).

Pollen is the key source of protein used for rearing brood, but it is only fed in small amounts to larvae. Rather, pollen collected by foragers is converted into stored bee bread. Consumption of bee bread stimulates the production of glandular secretions from the hypopharyngeal and mandibular glands to produce royal jelly or brood food, which is the major source of protein fed to developing larvae (Brodschneider and Crailsheim, 2010). In order to rear one larva, 25–37.5 mg of protein or the equivalent of 125–187.5 mg of pollen are needed (Hrassnigg and Crailsheim, 2005). When shortages in pollen stores arise, nurse bees respond by decreasing the number of visits to younger larvae, which are not yet largely invested with the colony's resources (Schmickl and Crailsheim, 2002). Another adaptive strategy to inadequate pollen stores is early capping of older larval cells (Schmickl and Crailsheim, 2001). Because capped brood no longer require feeding by the nurse bees, early capping reduces pollen demand. However, it is possible that reduced nurse visits or early capping could contribute to nutritional stress in larvae. It has been shown that starved worker larvae have an increased risk of developmental failure, which can lead to dwarf adults (Jay, 1963). Adult bees that were pollen stressed as larvae have also been shown to have lower body weight, forage precociously, forage less and for fewer days, waggle dance less frequently, and have a shorter life span compared to nest-mates reared with adequate pollen (Ament et al., 2010; Scofield and Mattila, 2015).

Adult bees, particularly those that are newly emerged and experiencing continued organ and glandular development and growth of fat bodies can experience poor development and decreased longevity due to diets low in pollen (Maurizio and Hodges, 1950). Also, drones that experience low-pollen conditions during the first few days of adulthood take longer to reach sexual maturity or do not reach it at all (Free and Williams, 1975).

Hormones play an important role in honey bee development as well. For example, JH prompts molting to occur, allowing for larval growth, and ensuring proper developmental progression (Fyg, 1959). Because proper functioning of the endocrine organs responsible for the distribution of hormones that trigger molting is dependent on larval nutrition, any impacts to nutrition could lead to problems during ecdysis (Fyg, 1959). These data indicate that perturbation of honey bee development due to inadequate access to food at any stage can negatively impact colony strength.

Additionally, as a compensatory survival mechanism in response to declines in pollen influx, adult bees may respond by cannibalizing brood rather than raising malnourished offspring (Schmickl and Crailsheim, 2004). Typically, larvae <3 d old are cannibalized, and the protein obtained is used to enrich brood food, which is fed preferentially to the remaining older brood (Schmickl and Crailsheim, 2001). Although, older larvae are spared, cannibalism would eventually lead to fewer adult bees, which could plausibly reduce the colony size for a period of time.

Finally, as discussed previously, foraging is the most dangerous and metabolically demanding task in a female honey bee's life. This role is typically reserved for older worker bees ranging in age between 21

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and 24 days (Seeley, 1982). In healthy colonies, forager mortality can exceed 15% per day (Russell et al., 2013). Because foraging bees are exposed to external stressors, they are normally at higher risk than hive bees to predation, chemical exposure, and weather events that lead to death. Hence, additional stress caused by perturbation of biological pathways leading to anomalies in foraging behavior, such as inability to avoid predation, disorientation, or poor homing capability could plausibly increase the likelihood of forager mortality. Overall, it is anticipated that reduced brood care, impaired development, and fewer bees in the colony, due to mortality at any life stage, could lead to a weakened colony.

3.22. KER12: abnormal role change within caste leads to weakened colony

Abnormal role change within caste leads to a weakened colony through a KER that is present in two AOPs (Fig. 1; AOP 2 and 4). Available literature has not explicitly evaluated this linkage in honey bee colonies, so the primary WoE for this KER is derived from biological plausibility.

3.22.1. Consideration of biological plausibility and empirical support

Studies comparing foraging bees and hive bees have begun to unravel interesting hormonal, physiological, and biochemical differences during behavioral development, particularly in preparation for flight and the transition to foraging. The unique adaptations observed in foraging bees, including, for example, decreased body mass, increased 10A troponin T isoforms (important muscle structural component), and increased metabolic flux capacity in the glycolytic pathway, could be targeted in future research comparing precociously foraging bees to normally developed foragers (Vance et al., 2009; Schippers et al., 2010; Schippers et al., 2006). These comparisons would provide a more thorough understanding of whether the expedited behavioral transition causes the poor flight performance and shortened lifespan observed in precocious foragers.

Due to the antioxidant properties of Vtg, the unique localization of Vtg in fat cells of the thorax and brain, and the flexible aging patterns of bees, studies have been conducted to understand the role of Vtg throughout the lifespan (Seehuus et al., 2006; Corona et al., 2007; Münch et al., 2015). By studying Vtg levels in various tissues from drones, workers, and queens of different ages with knockdown technology, it has been demonstrated that Vtg is expressed at higher levels in both the thorax and head of queens, with negligible and decreasing levels found in the tissues of worker bees as they age (Corona et al., 2007). Further, through evaluation of resistance to oxidative stress, studies show that Vtg plays a protective role in the bee by acting as a target of oxidative carbonylation and by scavenging free radicals (Seehuus et al., 2006). For example, Nelson et al. (2007) demonstrated that knockdown of Vtg significantly reduced honey bee lifespan. However, the relationship between Vtg and lifespan attributes is likely complex and influenced by numerous modulating factors. For example, differences in lifespan were identified upon knockdown of Vtg when comparing high and low pollen hoarding strains, where an increased lifespan was observed in the low hoarding strain (Ihle et al., 2015). Due to reductions of Vtg levels in foragers, it is biologically plausible that young bees, which transition to foraging precociously, also experience increased susceptibility to oxidative damage and, therefore, shortened lifespans, plausibly reducing the strength of the colony.

In addition to impacts on lifespan, studies evaluating performance of precociously-foraging bees have demonstrated poor hovering flight capacity, reduced flight span (distance and time), reduced number and duration of successful flights performed in the bee's lifetime, impaired olfactory reversal-learning, and a preference toward nectar (as opposed to pollen) foraging compared to bees that had completed a more normal, prolonged progression of behavioral development (Chang et al., 2015; Page et al., 2012; Perry et al., 2015). Bees that follow typical behavioral development proceed through dramatic physiological and biochemical changes (Vance et al., 2009). For example, accompanying the transition to foraging, bees undergo a 42% reduction in body mass, particularly in abdominal tissue (Vance et al., 2009).

It has been demonstrated that nurse bees hovering in air, due predominantly to their heavy bodies, were at or just below maximal attainable levels for wing stroke amplitude and wing angular velocity and were unable to maintain normal wingbeat frequency (Vance et al., 2009). Precocious foragers, which are also heavier than normal-aged foragers, likewise demonstrated an inability to maintain normal wingbeat frequency, therefore affecting flight performance (Vance et al., 2009; Chang et al., 2015). Structural changes also occur in flight muscles in preparation for foraging, allowing for metabolic enzymes to operate at higher capacity to support elevated metabolic rates compared to hive bees (Schippers et al., 2010). Thus, precocious foragers are not as physiologically-optimized for their role as normally-developing foragers. Such inadequacies could leave precocious foragers more prone to predation and unable to provide adequate amounts of food to the colony. Therefore, it is biologically plausible that these traits of the precocious forager could lead to a weakened colony.

In general, precocious foraging leaves fewer hive bees to tend to the developing brood. A reduction in hive bees could also contribute to a weakened colony, as the brood cannot be adequately fed, and optimal rearing temperatures cannot be maintained (Perry et al., 2015). The number of hive bees available to care for developing brood is another factor that dictates whether larvae receive adequate nutrition. Studies have demonstrated that higher worker-to-larvae ratios are associated with increased lifespan and dry weight of offspring (Eischen et al., 1983). And, as described in KER11, inadequate nutrition during development has detrimental effects to the colony as well.

3.22.2. Weight of evidence consideration for KER12

The motive for honey bees to have developed the compensatory strategy of precocious foraging suggests that accelerated behavioral transition, under ideal conditions, must lead to strengthening of the colony (even if temporarily weakened) and therefore survival. A number of studies have been conducted to understand the changes in the precocious forager's physiology and inability to thrive, as well as modeling efforts to predict colony-level outcomes based on precocious foraging over time. But because precocious foraging in the honey bee is an adaptive response to overcome the stress of starvation or bee losses (including losses from swarming), it would be equally advantageous to understand the conditions under which precocious foragers are successful in replenishing the food supply without overall detrimental effects to the developing brood or adults.

Therein lies the greatest uncertainty surrounding the KER linking role change within caste to a weakened colony. Questions as to when and how the upstream KE leads to beneficial versus adverse effects at the colony level have yet to be addressed; thus, our WoE assignment for this KER is 'weak.' Research initiatives, focused on evaluation of conditions that lead to strength and survival, in addition to long-term colony-level effects over time (seasonal effects) following accelerated foraging, would be important.

3.23. KER13: impaired hive thermoregulation leads to weakened colony

The KER linking impaired hive thermoregulation to weakened colony is found in one AOP description (Fig. 1; AOP 4). The importance of thermoregulation for both the developing brood and overall colony has been documented in previous sections. However, only a limited number of studies directly explore the link between thermoregulation and overall colony strength. Therefore, the WoE for this KER is based primarily on biological plausibility.

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3.23.1. Consideration of biological plausibility and empirical support

An optimal hive temperature of ~35 °C is required for both adult and developing bees to maintain adequate body temperatures. Adult bees can experience impaired muscular performance when thorax temperature falls below ~28 °C, and experience chill coma (i.e., muscular paralysis) at temperatures below 12 °C. Chill coma results in death of the bee if low temperature is experienced for an extended duration. Additionally, honey bee larvae and pupae are stenothermic, meaning they depend on strict temperature regulation for proper development (Stabentheiner et al., 2010). Deviations from the optimal hive temperature range can lead to crippling or death of the developing bee (Fyg, 1959). One of the primary tasks of the hive bee involves maintaining optimal brood rearing temperature and humidity. Nurse bees help regulate excessive temperatures of the brood nest employing techniques such as fanning their wings or spreading water droplets within and on top of brood cells (Southwick and Heldmaier, 1987). Additionally, during cooler seasons, hive members form a cluster and utilize shivering thermogenesis to produce heat and maintain optimal hive temperatures. Southwick (1985) demonstrated that at winter temperatures, honey bee metabolism increased as the amount of bees in a cluster increased. Further, larger clusters were better insulated than smaller clusters (≤5 g), which were unable to survive 2 °C temperature overnight (Southwick, 1985). Impaired hive thermoregulation, plausibly, could result in bee mortality and/or impaired development, effectively weakening the colony.

3.24. AO: colony, loss/failure

Colony death/failure is the AO for all six of the developed AOPs (Fig. 1) and occurs at the colony level of biological organization which would imply probable population level outcomes. Colony death/failure is defined as demise of a functional colony. Dramatic losses in the number of managed honey bee colonies have been reported across the globe (Potts et al., 2010) and efforts have been undertaken to survey and identify trends in losses over time, particularly in the US and European Union. Most recent survey results collected in the US have shown that managed honey bee colony losses are significantly higher than those deemed acceptable by beekeepers (Seitz et al., 2015). From surveying commercial (>300 colonies), sideline (25–300 colonies), and small scale (<25 colonies) beekeepers, average annual colony losses (both summer and winter losses) per operation in the US during 2014–2015 were 49%, compared to 18.7% that has been identified by beekeepers as an acceptable loss rate (Seitz et al., 2015). Relevant to the AOPs described here, starvation, poor over-winter survival, and weak colonies, were among the most common perceived causes of loss reported by bee keepers (Seitz et al., 2015). Commercial beekeepers, managing thousands of colonies, self-reported colony collapse disorder and pesticides as third and fourth leading reasons for colony loss, respectively (Seitz et al., 2015).

3.25. KER14: weakened colony leads to colony death/failure

The KER linking weakened colony to colony death/failure is critical to all six AOP descriptions (Fig. 1; AOP 1 to 6). A weakened colony can be manifested as loss of bees at any life stage and/or impaired brood development, although the threshold for the number of losses or degree of abnormal development experienced by the overall colony that would lead to certain loss or failure of the hive is difficult to determine.

3.25.1. Consideration of biological plausibility and empirical support

The loss or failure of a honey bee colony is a result of a decrease in the number of healthy bees available to maintain all essential tasks of the colony. Essential tasks include caring for the queen and brood, hygienic behavior, effectively foraging and distributing an adequate supply of food to colony members, and maintaining optimal hive temperature. Therefore, impairment of any one of these tasks can be detrimental to the colony, particularly if sustained for a prolonged period or if other stressors are impacting colony health simultaneously. Bee deaths are a result of a number of different factors and can occur rapidly or over an extended period of time. Therefore, it is critical to evaluate and understand the impact of stressors to colony strength over time and the ability or inability of the colony to compensate for bee losses. Models evaluating colony dynamics have provided estimates for honey bee death rates that could lead to colony loss. For example, Becher et al. (2010) developed a model that used a simulation of a temperature gradient across a brood comb, which was informed by empirically measured hive temperatures, to understand the impact of brood temperature on the age at first forage. Results from this model indicated that colony survival was greatly dependent on the number of bees available for heating the hive (Becher et al., 2010). Another model by Thompson et al. (2005), which considered impacts of pesticides, seasonal aspects of queen egg-laying, and adult mortality rates indicated that impacts on the size of the colony over winter is a critical determinant of colony survival (Thompson et al., 2005). These models tend to provide support that available workers and colony size are important for colony survival. As discussed in KER8, a model developed by Khoury et al. (2011) and based on mathematical relationships of worker bee labor division and bee longevity and colony growth, takes into account precocious foraging and their known reduced lifespan. This model suggests that if forager death rates are high and sustained the colony can rapidly fail (Khoury et al., 2011). Alternatively, if the forager death rate is high and hive bees forage precociously, the number of foragers may be restored given ample availability of forage, although the overall adult lifespan is reduced (Khoury et al., 2011). Therefore, the reduced lifespan leads to reduced contributions to colony growth and brood care, ultimately reducing the colony's brood-rearing capacity. Accounting for the dynamics of the colony, this model predicts that reduction of the foraging force by approximately two-thirds will put the colony at risk of failure (Khoury et al., 2011). Another honey bee model, BEEHAVE, takes into account colony dynamics, foraging landscape and performance, bee development, and impacts of the Varroa mite and Varroa-transmitted viruses, allowing for evaluation of multiple-stressor scenarios (Becher et al., 2014). Due to the difficulty in actually testing different multi-stressor scenarios at the colony level, such models provide the biologically plausible predictions of honey bee colony dynamics that can be useful for managing and understanding where a weakened colony, caused by multiple factors, may lead to colony death/failure.

4. Discussion

The aim of this review and analysis was to organize available literature utilizing the AOP framework to capture the state-of-science linking perturbation (e.g., via neonicotinoids) of the honey bee nAChR to changes in biology, spanning different levels of organization, culminating in colony level impacts. This work was conducted not only to better understand the mechanism through which nAChR agonists, such as neonicotinoids, might contribute to honey bee colony death, but to lay the foundation, through the development of KE/KER descriptions and WoE evaluation, for future research and continued evolution of an AOP network. Such information could be used to more holistically evaluate impacts of both chemical and non-chemical stressors on these critical pollinators. To facilitate this type of collaboration, all AOPs described in this paper were entered into the AOPWiki (https://aopwiki.org/), a publically available AOP development platform that uses crowd-sourcing to capture AOP knowledge (Supplemental Materials, Table S1).

4.1. Taxonomic relevance of AOPs to native bees

Taxonomic relevance is a consideration in AOP development, which aims to address the question of how broadly a defined AOP can be extrapolated across species. In the context of the AOPs described herein, they are primarily focused on honey bees. But there are also concerns for declining or disappearing unmanaged wild and native bee species

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including bumble bees, stingless, and solitary bees (Goulson et al., 2015; Tomé et al., 2012; Mommaerts et al., 2010; Cameron et al., 2011; Sandrock et al., 2014). Pesticides, including neonicotinoids, have been identified as potentially contributing to the noted declines, possibly through many of the same KEs that were developed for the AOPs initiated via activation of the nAChR (Mommaerts et al., 2010; Wu-Smart and Spivak, 2016). From the AOPs described herein, upstream KEs are more likely to be conserved across a majority of bee species than downstream events, which may unfold differently due to, for example, variations in colony structure, including a complete lack thereof in most species. That said, evidence from a previous cross species comparison of honey bee nAChR sequence similarity to other taxa, using the US Environmental Protection Agency Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS; https://seqapass.epa.gov/seqapass/) tool, indicated that nAChR subunits, and ligand binding domains within subunits, are highly similar among bee species, including those in the genus *Apis*, *Bombus*, *Megachile*, *Melipona* and *Habropoda* (LaLone et al., 2016). Therefore, conservation of the MIE and KE for nAChR activation and desensitization, respectively, provides a line of evidence to support extrapolation of these early events in the pathway to other bee species. With the identification of protein targets involved in various KEs along the developed AOPs we intend to follow-up with additional SeqAPASS evaluations to more thoroughly define the taxonomic domain of applicability throughout the AOP network. However, it is important to note that, although binding sites may be conserved, other variables, including natural history and differences in foraging behaviors, may ultimately dictate relative species susceptibility and possible adverse outcomes (Thompson and Hunt, 1999; Decourtye and Devillers, 2010).

4.2. Identification of knowledge gaps

An evaluation of the WoE for KERs was conducted to define the strength of the relationships linking upstream to downstream KEs and additionally identify critical knowledge gaps or uncertainties that could be used to guide future research efforts. Uncertainty identified from evaluation of the available literature pertaining to the MIE (nAChR activation), KE1 (nAChR desensitization), KE2 (mitochondrial dysfunction), and KE3 (Ca^{2+}-calmodulin activated signal transduction, altered) stem from data gaps surrounding characterization of the nAChR subunits in insects, experimental or natural agonist exposure scenarios that lead to honey bee nAChR desensitization, impacts of prolonged desensitization of the nAChR to Ca^{2+} signaling and downstream neuronal signaling events, and localization of the nAChR on invertebrate mitochondria. Research to explore these topics is important to further understanding the mechanisms through which nAChR agonists, such as neonicotinoids, might lead to impacts on honey bee learning and memory. If designed properly, such studies would also aid in defining the quantitative relationships that define what levels of a pathway perturbation may be adverse in the context of multiple stressors acting on the colony.

From evaluation of the WoE linking abnormal role change within caste (KE6) to colony weakened (KE8), we identified that uncertainty exists as to how brood care may be impacted by the early departure of hive bees to forage precociously or, conversely, whether forager bees that have reverted to hive bees may impact brood care and further larval development. Studies that assess brood health and development over time when reared under circumstances that lead to abnormal shifts in the worker bee role would aid in substantiating models that predict colony losses due to precocious foraging (Perry et al., 2015). Further, since both precocious foraging and reversion of foragers to hive activities are compensatory mechanisms to fill critical roles in the colony, it would be anticipated that under certain circumstances, abnormal role changes within the caste could lead to improved colony strength and survival. Studies aimed at understanding scenarios in which such role changes are beneficial versus when they are detrimental (perhaps when multiple stressors are impacting the colony or time of season when the event occurred) would add to our understanding of the KER.

4.3. AOP network

The six AOPs described in this work assemble into a putative AOP network that may more realistically represent the biological implications of interactive stressors on the honey bee colony (Fig. 4A). The focus of this initial effort was to establish a network based on the MIE for specific chemical initiators, the neonicotinoids, with the goal of describing KEs that would likely be re-used and expanded upon in future AOP development for both chemical and non-chemical stressors impacting honey bee health. It is recognized that even when considering activation of the nAChR there are other AOPs that could be, and subsequently should be, described. As examples, altered Ca^{2+}-calmodulin activated signal transduction could also lead to impacts on mobility that could affect brood care, hygienic behavior, food processing, etc., or reduced pollen stores from abnormal foraging could affect queen egg laying activity, however these additional AOPs are yet to be described (Thiel and Köhler, 2016; Wu-Smart and Spivak, 2016). Therefore, the foundational AOP work described here on honey bees was conducted to facilitate further collaboration and additional AOP development by making it less daunting for other researchers to build on predefined KEs and KER descriptions. It has been suggested that AOP networks are necessary to evaluate multi-stressor perturbations (e.g., Hooper et al., 2013), as it is widely recognized that stressors encountered by honey bees in the environment do not act in isolation.

Over 100 pesticides have been detected in honey bees and hive products, indicating that multiple chemical exposures occur with the potential for numerous interacting effects (Mullin et al., 2010). For example, Palmer et al. (2013) described additive adverse effects on neuronal function in Kenyan cells from isolated honey bee brains following combined exposure to both neonicotinoids and an organophosphate. Although these pesticides have different molecular actions, i.e., nAChR activation and acetylcholinesterase inhibition, the pathways converge at the level of the neuron likely leading to the observed additive effect (Palmer et al., 2013). For example, Russom et al. (2014) developed an AOP describing perturbation by chemical stressors, such as organophosphate and carbamate insecticides, that act by directly inhibiting acetylcholinesterase, leading to acute mortality. Acetylcholinesterase is an enzyme involved in the termination of neurotransmission via hydrolysis of the neurotransmitter acetylcholine. Therefore, upon perturbation of this enzyme by a chemical stressor, acetylcholine accumulates in the synapse, which leads to abnormal activation of cholinergic receptors, including nAChR (as acetylcholine is its natural ligand) (Russom et al., 2014). Because these pathways converge on a common KE, activation of the nAChR, the upstream events beginning with acetylcholinesterase inhibition, could be added to the developed AOP network. Other chemical stressors, particularly pesticides that act on molecular targets involved in neurotransmission, also are likely to converge on the upstream KE for altered Ca^{2+}-calmodulin activated signal transduction (Fig. 4B). Likewise, non-chemical stressors can also contribute to colony failures. A few examples of putative AOPs (AOPWiki; https://aopwiki.org/), capturing effects of non-chemical stressors such as Varroa mite infestation, Nosema spp. infection, forage availability, or weather events likely to impact downstream KE such as abnormal foraging activity and behavior or weakened colony (Fig. 5), were incorporated into the overall AOP network, allowing better consideration of the cumulative impacts of a range of chemical and non-chemical influences on colony survival.

A potentially critical component of the honey bee colony that was not explicitly considered in the current AOP development effort involved perturbations leading to adverse effects on queen and drone health. Certainly, if egg-laying by the queen is decreased and or drone mating, sperm quality or abundance is reduced, the overall strength and reproductive ability of the colony will be diminished. Therefore, as the AOP network expands it will become more feasible to evaluate the pleiotropic effects of multiple concurrent stressors that may contribute to honey bee colony losses.

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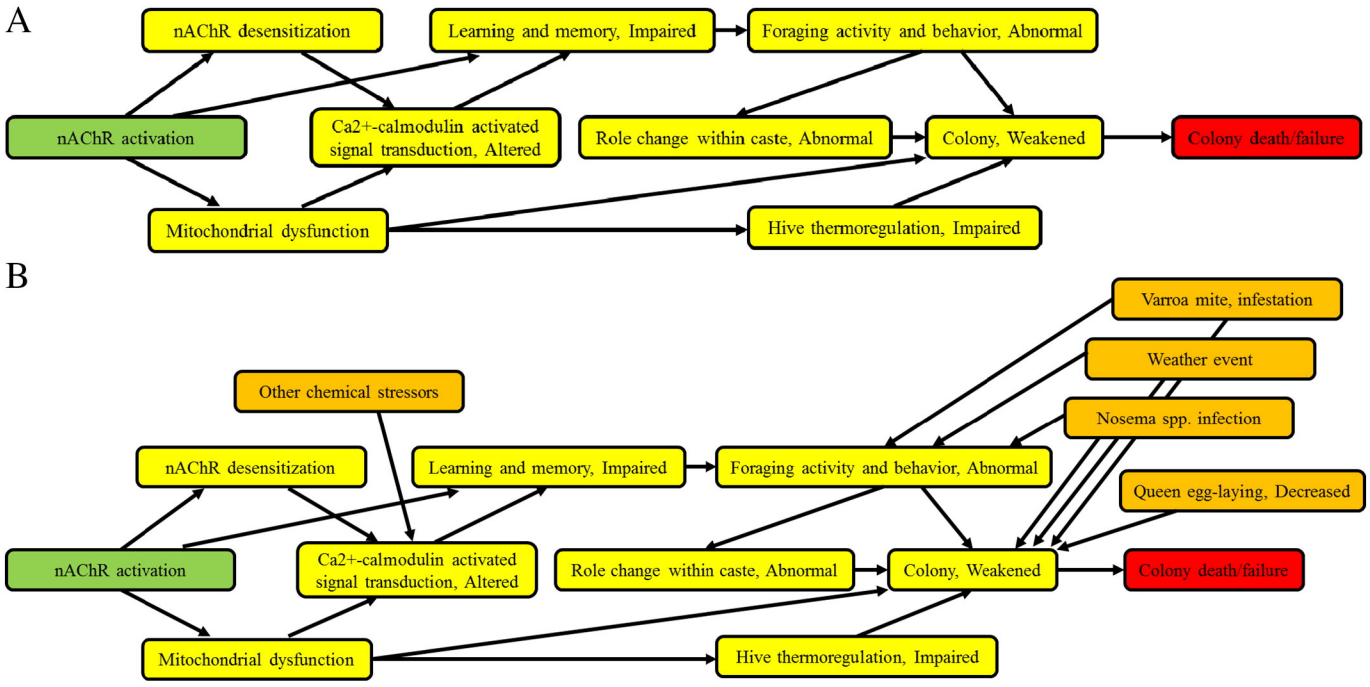

Fig. 4. Adverse outcome pathway networks describing perturbation via the nicotinic acetylcholine receptor (nAChR) (A) and including additional stressors (B) from the putative AOPs illustrated in Fig. 5. Green box represents the defined molecular initiating event. Yellow boxes represent defined key events. Orange boxes represent other chemical and non-chemical stressors, and red box indicates the adverse outcome of regulatory concern. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

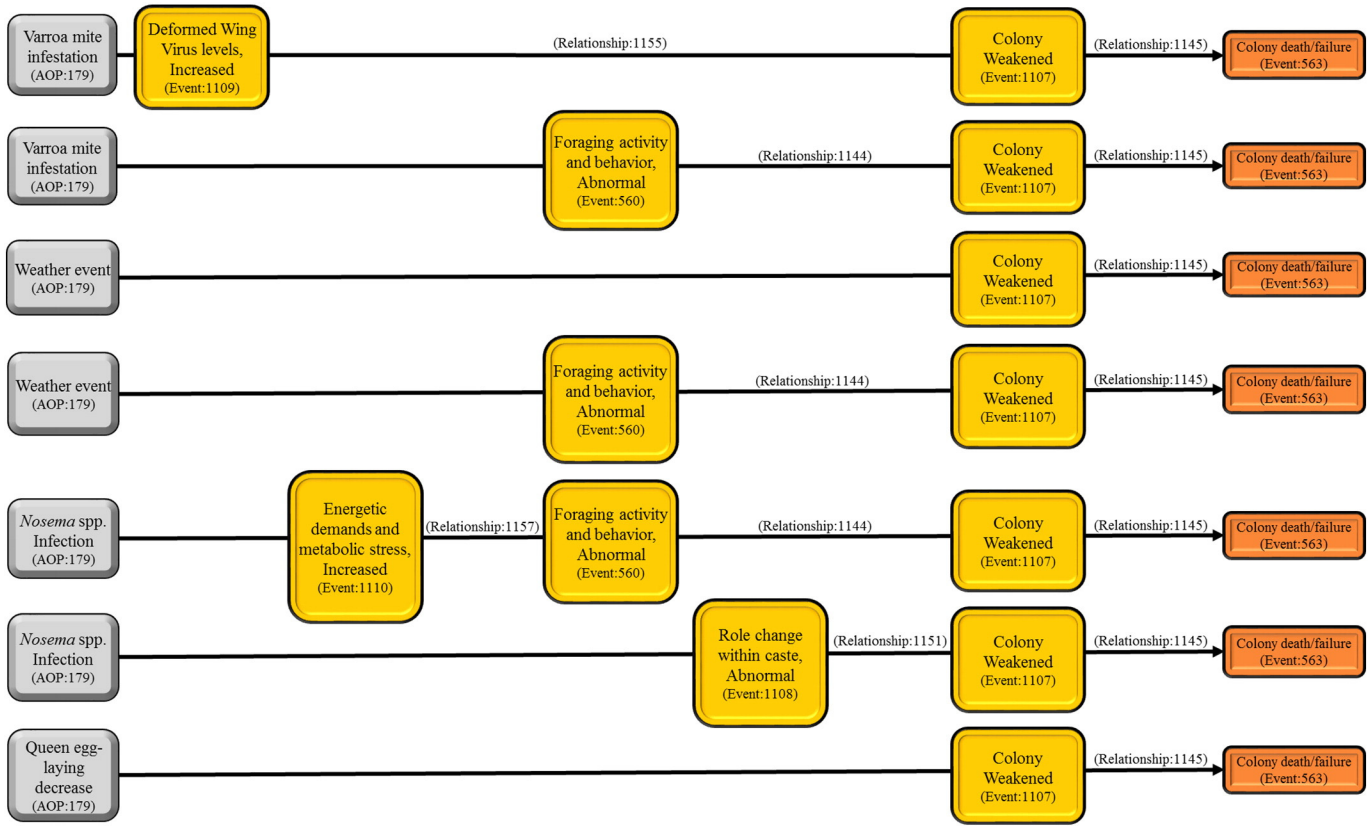

Fig. 5. Putative (hypothesized) adverse outcome pathways delineated based on four common non-chemical stressors. Grey box indicates the non-chemical stressor, yellow box indicates key events and red box the adverse outcome. Note that three key events and the adverse outcome, depicted here, were defined by the chemical stressor AOPs. Found in parentheses are the unique AOP, Event, and Relationship identifiers as references for the AOPWiki (AOPWiki; https://aopwiki.org/) where these putative AOPs have been entered. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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An important benefit of a network-based analysis is the possibility of identifying biological nodes that are susceptible to multiple stressors and can therefore be used to guide targeted mitigation strategies. That is, when more than one upstream KE or MIE converge on a single downstream KE, such as altered Ca^{2+}-calmodulin activated signal transduction (KE3) or colony weakened (KE8), interactive effects may be predictable. Due to the knowledge that neonicotinoids and, potentially, other neuroactive insecticides are likely to converge on altered Ca^{2+}-calmodulin activated signal transduction (KE3) as a common node (Fig. 4A and B), it would seem logical to mitigate impacts by increasing public awareness pertaining to appropriate application of insecticides operating via those nodes to reduce unnecessary exposure to bees and other beneficial insects. Further, with multiple stressors likely to adversely impact foraging activity and behavior (KE5) and weaken the colony (KE8), another pragmatic approach to mitigation would be to facilitate foraging success and improve colony strength by increasing the availability of pollinator habitat, with the goal of making access to adequate and nutritionally diverse floral resources readily available throughout the growing season.

Some initiatives along these lines have already been proposed and adopted in attempts to curb the impacts of stressors to honey bee colonies and other beneficial pollinators. In 2014, the Office of the President of the US released a Presidential Memorandum that led to the development of a Pollinator Health Task Force, consisting of leadership from Federal Agency partners, and the development of a National Strategy to Promote the Health of Honey Bees and Other Pollinators in 2015 (https://www.whitehouse.gov/blog/2015/05/19/announcing-new-steps-promote-pollinator-health). This strategy document included the Pollinator Research Action Plan to address uncertainties associated with pollinator health and colony survival in the US. Among numerous outlined initiatives, these communications set goals to stimulate public engagement, awareness, and action on topics related to pollinators, including pesticide application and more thorough evaluation of their potential impacts, developing tools to reduce impacts from pests and pathogens, as well as to initiate efforts to increase the quantity and quality of pollinator habitat.

In conclusion, from AOP development focused on perturbation of the honey bee nAChR, AOP network construction, and WoE evaluation, sufficient evidence indicates that a connection is plausible to link activation of nAChR to colony death/failure. However, quantitative linkages involving a variety of modulating factors and uncertainties (i.e., subunits that make up an insect nAChR, presence of nAChR in invertebrate mitochondria, etc.) make it difficult to define exactly what concentration, duration, and/or timing of neonicotinoid exposure and interaction with nAChR will lead to adverse colony outcomes. Therefore, the AOP framework and overall network can be used to guide future research to quantitatively assess such relationships.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.01.113.

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Supplemental Materials, Table S1. AOPWiki Pages

[a]Adverse Outcome Pathway	Key event	Description	Triggers
1 (AOP:88)	nAChR activation (Event:559)	Leads to (Relationship:1146)	Learning and memory, impairment (Event:341)
	nAChR activation (Event:559)	Leads to (Relationship:658)	nAChR desensitization (Event:663)
	nAChR desensitization (Event:663)	Leads to (Relationship:1309)	Ca^{2+}-calmodulin activated signal transduction, altered (Event:1243)
	Ca^{2+}-calmodulin activated signal transduction, altered (Event:1243)	Leads to (Relationship:1310)	Learning and memory, impairment (Event:341)
	Learning and memory, impairment (Event:341)	Leads to (Relationship:383)	Foraging activity and behavior, abnormal (Event:560)
	Foraging activity and behavior, abnormal (Event:560)	Leads to (Relationship:1144)	Colony, Weakened (Event:1107)
	Colony, Weakened (Event:1107)	Leads to (Relationship:1145)	Colony death/failure (Event:563)
2 (AOP:89)	nAChR activation (Event:559)	Leads to (Relationship:1146)	Learning and memory, impairment (Event:341)
	nAChR activation (Event:559)	Leads to (Relationship:658)	nAChR desensitization (Event:663)
	nAChR desensitization (Event:663)	Leads to (Relationship:1309)	Ca^{2+}-calmodulin activated signal transduction, altered (Event:1243)
	Ca^{2+}-calmodulin activated signal transduction, altered (Event:1243)	Leads to (Relationship:1310)	Learning and memory, impairment (Event:341)
	Learning and memory, impairment (Event:341)	Leads to (Relationship:383)	Foraging activity and behavior, abnormal (Event:560)
	Foraging activity and behavior, abnormal (Event:560)	Leads to (Relationship:1150)	Role change within cast, abnormal (Event:1108)
	Role change within cast, abnormal (Event:1108)	Leads to (Relationship:1151)	Colony, Weakened (Event:1107)
	Colony, Weakened (Event:1107)	Leads to (Relationship:1145)	Colony death/failure (Event:563)
3 (AOP:77)	nAChR activation (Event:559)	Leads to (Relationship:1146)	Learning and memory, impairment (Event:341)
	nAChR activation (Event:559)	Leads to (Relationship:581)	Mitochondrial dysfunction (Event:177)
	Mitochondrial dysfunction (Event:177)	Leads to (Relationship:1311)	Ca^{2+}-calmodulin activated signal transduction, altered (Event:1243)
	Ca^{2+}-calmodulin activated signal transduction, altered (Event:1243)	Leads to (Relationship:1310)	Learning and memory, impairment (Event:341)
	Learning and memory, impairment (Event:341)	Leads to (Relationship:383)	Foraging activity and behavior, abnormal (Event:560)
	Foraging activity and behavior, abnormal (Event:560)	Leads to (Relationship:1144)	Colony, Weakened (Event:1107)
	Colony, Weakened (Event:1107)	Leads to (Relationship:1145)	Colony death/failure (Event:563)
4 (AOP:87)	nAChR activation (Event:559)	Leads to (Relationship:1146)	Learning and memory, impairment (Event:341)
	nAChR activation (Event:559)	Leads to (Relationship:581)	Mitochondrial dysfunction (Event:177)
	Mitochondrial dysfunction (Event:177)	Leads to (Relationship:1311)	Ca^{2+}-calmodulin activated signal transduction, altered (Event:1243)
	Ca^{2+}-calmodulin activated signal transduction, altered (Event:1243)	Leads to (Relationship:1310)	Learning and memory, impairment (Event:341)
	Learning and memory, impairment (Event:341)	Leads to (Relationship:383)	Foraging activity and behavior, abnormal (Event:560)
	Foraging activity and behavior, abnormal (Event:560)	Leads to (Relationship:1150)	Role change within cast, abnormal (Event:564)
	Role change within cast, abnormal (Event:564)	Leads to (Relationship:1151)	Colony, Weakened (Event:1107)
	Colony, Weakened (Event:1107)	Leads to (Relationship:1145)	Colony death/failure (Event:563)
5 (AOP:79)	nAChR activation (Event:559)	Leads to (Relationship:581)	Mitochondrial dysfunction (Event:177)
	Mitochondrial dysfunction (Event:177)	Leads to (Relationship:1152)	Hive thermoregulation, impaired (Event:568)
	Hive thermoregulation, impaired (Event:568)	Leads to (Relationship:1153)	Colony, Weakened (Event:1107)
	Colony, Weakened (Event:1107)	Leads to (Relationship:1145)	Colony death/failure (Event:563)
6 (AOP:178)	nAChR activation (Event:559)	Leads to (Relationship:581)	Mitochondrial dysfunction (Event:177)
	Mitochondrial dysfunction (Event:177)	Leads to (Relationship:1154)	Colony, Weakened (Event:1107)
	Colony, Weakened (Event:1107)	Leads to (Relationship:1145)	Colony death/failure (Event:563)

[a]AOP number assignment from Figure 1

All parentheses are the unique IDs for AOPs, Events, and Relationships designated in the AOPWiki as of October 13th, 2016