**Title:** Predator-induced stress responses in insects: A review spanning from endocrine to cellular responses

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**Highlights:**

- Predation risk stimulates the octopamine-adipokinetic hormone (OAH) axis in insects – an analogue to the vertebrate hypothalamo-pituitary-adrenal (HPA) axis in vertebrates.
- Studies should account for taxon- and sensory-specific differences in prey responses
- The insect stress response to predation mirrors that observed in vertebrates
- A physiological network perspective helps compare responses across insect predator-prey systems
- Best practices for studying predator-induced stress in insects are presented

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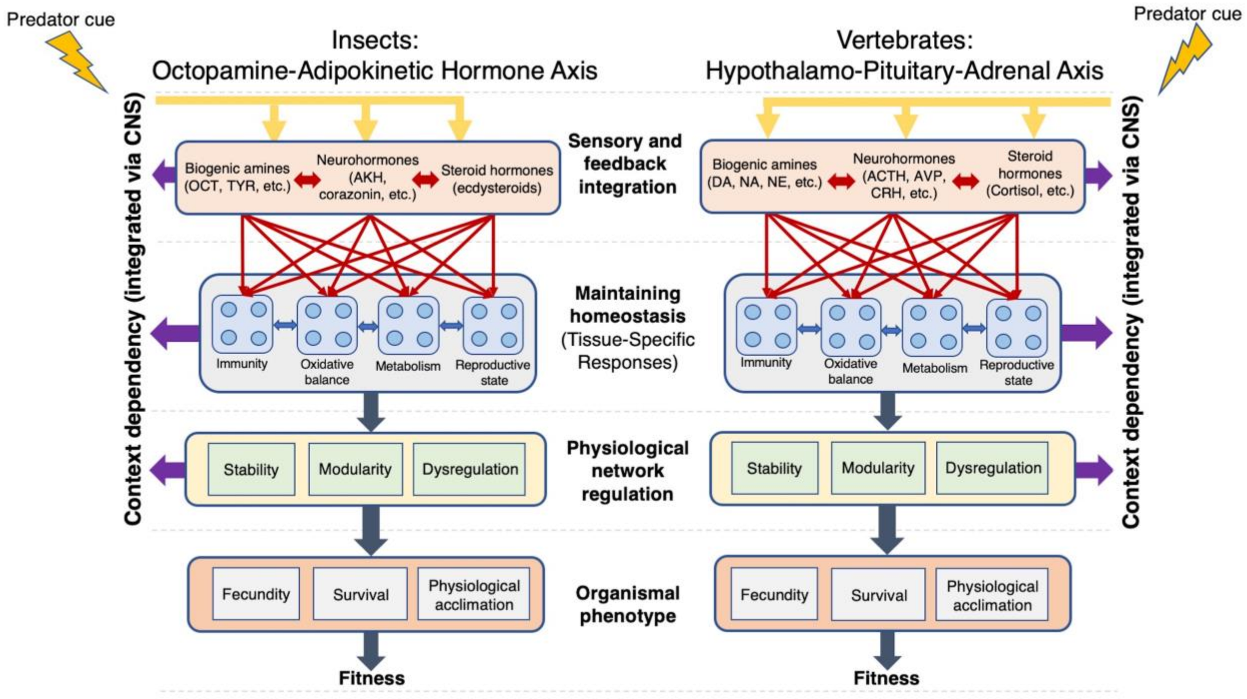
## Abstract

Predators can induce extreme stress and profound physiological responses in prey. Insects are the most dominant animal group on Earth and serve as prey for many different predators. Although insects have an extraordinary diversity of anti-predator behavioral and physiological responses, predator-induced stress has not been studied extensively in insects, especially at the molecular level. Here, we review the existing literature on physiological predator-induced stress responses in insects and compare what is known about insect stress to vertebrate stress systems. We conclude that many unrelated insects share a baseline pathway of predator-induced stress responses that we refer to as the octopamine-adipokinetic hormone (OAH) axis. We also present best practices for studying predator-induced stress responses in prey insects. We encourage investigators to compare neurophysiological responses to predator-related stress at the organismal, neurohormonal, tissue, and cellular levels within and across taxonomic groups. Studying stress-response variation between ecological contexts and across taxonomic levels will enable the field to build a holistic understanding of, and distinction between, taxon- and stimulus-specific responses relative to universal stress responses.

**Keywords:** adipokinetic hormone; predator-prey; neurohormone; octopamine; cellular effector

## Graphical Abstract



Note: Conceptual framework in graphical abstract adapted from Cohen et al. (2012).

## Introduction

Over the last century, major advances in animal behavior and physiology have been made through research on predator-prey interactions. In particular, predator-prey research has contributed significantly to behavioral and landscape ecology, leading to the recognition that nonlethal, indirect interactions between predators and prey can influence prey demography and community interactions as much as direct mortality (Laundré et al., 2014). Specifically, predators are capable of influencing prey optimal foraging patterns, behavioral tactics within game theory strategies (Gross, 1996), and habitat use, among many other phenomena (Brown et al., 1999; Laundre et al., 2010). Studies on predator and prey physiology have made it possible to quantify the state of at-risk animal populations or species using molecular evidence, and the field of conservation resource management has recently incorporated approaches to measuring the strength of apex predation in a given habitat using parameters of prey physiology alone (Ferrer and Zimmer, 2013; Leroux et al., 2012; Sheriff and Thaler, 2014). Laboratory studies, primarily involving vertebrates, have teased apart physiological mechanisms and downstream responses of predator-related auditory, olfactory, visual, and tactile cues (Clinchy et al., 2013; Sabet et al., 2015; Adamo et al., 2013; Miller et al., 2014). When prey encounter a hunting predator, a stress response initiates many downstream changes. Key molecular pathways that regulate cellular stress responses to predator stimuli initiate typical organismal stress responses, such as increased respiration and modified behavior. Due to their well-studied nature, vertebrate predator-prey systems provide a template for understanding the neurohormonal signaling, physiology, life history, and behavior of insect prey (Stenzel-Poore et al. 1992).

Insects dominate Earth with more than 5 million species (Stork et al., 2015), and they serve as primary prey for many predators. However, molecular mechanisms of predation-related stress in insects are poorly studied (Adamo, 2017; Farooqui, 2012; Huising and Flik, 2005). It is now thought that an ancestral stress-response system shared among animals predates the protostome/deuterostome divergence (Huising and Flik, 2005; Misof et al., 2014; Roeder, 2005), which suggests that there may be similarities in the insect and vertebrate systems found today. Despite this shared inheritance, insects exhibit an extraordinary diversity of life histories, niches, and morphologies that have resulted in the evolution of distinct, sensory-specific molecular signal transduction mechanisms tied to particular sensory structures (Benton et al., 2009; Rimal and Lee, 2018). Due to this profound diversity among insects, recent advances in molecular sequencing and knockdown applications have demonstrated the need to account for taxon-, sensory-, and tissue-specific divergences in molecular physiology when analyzing predator-induced insect stress responses, even within a single insect family (Lam et al., 2013).

A first step toward understanding insect stress responses is to contrast them with the well-studied vertebrate stress response model. Although substantial advances have been made (Adamo and Baker, 2011; Kodrík et al., 2015) in comparison to vertebrates, the molecular pathways and downstream effectors that play a role in predator-induced stress responses of insects are under-studied or unknown. This review synthesizes the literature on physiological predator-induced stress responses in insects from sensory perception and neurohormonal signaling through organismal responses. We highlight some of the model insect species that have been studied, note the challenges of conducting integrative studies of stress responses given the great diversity of insects and their stress responses, and recommend future directions that may help disentangle these complicated predator-induced responses of insect prey.

## A comparison of invertebrate and vertebrate stress physiology

Comparative studies in stress biology can reveal striking homologies, instances of convergent evolution, and stark differences in the way animal species cope with biotic and abiotic disturbances. The vertebrate hypothalamo-pituitary-adrenal (HPA) axis is one of the most well-studied, stress-induced physiological frameworks in biology (Harris and Carr, 2016). Study of the HPA axis has broadened our understanding of diverse physiological responses and neurobehavioral disorders, including post-traumatic stress disorder in humans (Clinchy et al., 2013; Roszkowski et al., 2016; Zanette and Clinchy, 2017) and stimulus habituation in laboratory rats and mice (Takahashi et al., 2005). The HPA axis is comprised of a series of neurohormonal cascades that cause neurosecretory and endocrine cells to release chemicals, including neurotransmitters, neuropeptides, and protein hormones, into the bloodstream during times of stress and bodily exertion (e.g. during predator-prey interactions; Fleshner et al., 2004; Fig. 1).

Stress-related hormones induce a variety of often species-specific physiological responses throughout the body, such as the transport of energy-rich molecules to target tissues (Harris and Carr, 2016), increases in heart rate and respiration (Zollinger et al., 2011), and behavioral modifications (Breviglieri et al., 2013), all of which can impact fitness (Bonier et al., 2009). Although the HPA axis and its effects on vertebrate cellular- and organ-level physiology in the context of predation risk are well-studied, a similarly well-defined model describing the stress-induced regulation of cellular responses in insects and other arthropods remains nascent.

Over the past several decades, organismal (Hack, 1997; Keiser and Mondor, 2013), transcriptional (Aruda et al., 2011; Cinel and Taylor, 2019), and cellular effector (Even et al., 2012; Slos and Stoks, 2008; Van Dievel et al., 2016) responses have been reported in insects both during and after exposure to predators. However, the specific molecules, cell types, and tissues involved in predator-induced stress responses in insect prey, as well as similarities among insect systems have not been extensively reported. In particular, taxonomic and experimental biases associated with the entomological literature may contribute to our limited knowledge in this field. Octopamine (OCT) and adipokinetic hormones (AKH) represent some of the best-studied neurohormones in insects, and several lines of evidence indicate parallels between the OCT-AKH axis and the vertebrate HPA axis. We discuss the functions and diversity of OCT- and AKH-induced responses and compare them to the vertebrate HPA axis in detail in the following sections.

## Neuroendocrine pathways and molecular messengers in stressed insects

To contend with diverse and dynamic conditions, insects possess a suite of neurohormonal cascades that adjust organismal physiology during times of exertion. Most research on insect stress-responsive neurohormonal signaling has focused on the biogenic monoamines OCT and tyramine (Davenport and Evans, 1984; Farooqui, 2012; Jones et al., 2011; Roeder, 2005), as well as a family of small neuropeptides known as AKHs (Adamo, 2017a; Even et al., 2012; Gäde, 2009; Kodrík et al., 2015; Orchard et al., 1993). OCT, a neurogenic relative of the vertebrate neurotransmitter norepinephrine (Verlinden et al., 2010), is thought to be the “first responder” during acute stress reactions in insects and acts as a neurohormone, neuromodulator, and neurotransmitter (Farooqui, 2007; Orchard, 1981). Notably, OCT and AKH are also intimately involved in the insect immune response. OCT and AKH are often measured at high hemolymph concentrations following a lesion, bacterial infection, and even parasitism (Adamo, 2017, 2012). Some researchers hypothesize that an ancestral molecular component of the stress and immune responses predating the vertebrate/invertebrate divergence has since been modified into octopamine in invertebrates and norepinephrine in vertebrates (Adamo, 2014; Farooqui, 2012; Ottaviani and Franceschi, 1996; Roeder, 2005). Although not predating the protostome-deuterostome split, Li et al. (2016) also provide evidence that AKH and its associated G-protein coupled receptors emerged in ancestral Lophotrochozoans around 550 Mya and show that AKH is an ancient, well-conserved component of invertebrate neurophysiology.

Recent work has focused on explaining how seemingly maladaptive physiological responses, particularly to prolonged stressors, in vertebrates and invertebrates could have evolved under natural selection. Adamo (2017) proposes a context-dependent tradeoff between the metabolically costly insect stress and immune responses, both of which are at least partially activated by OCT and AKH signaling. Both OCT and AKH trigger the fat body to release lipids into the hemolymph, where they are then loaded onto lipoproteins, molecules that also can play a functional role during immune surveillance (Adamo, 2017). Adamo (2017) also notes the sharing, reconfiguration, and borrowing of several other molecular resources among the extra- and intra-cellular signaling cascades and cellular effectors induced by both the immune and stress systems. Finally, Adamo (2017) argues that this shared function of one molecular resource, specifically Apolipophorin III (ApoLpIII), between two physiological contexts explains why reduced antioxidant activity, cell-specific metabolic exhaustion, and immune inhibition commonly co-occur during periods of chronic stress.

OCT acts as a neurohormone, neuromodulator, and neurotransmitter in both vertebrates and invertebrates (Farooqui, 2007; Orchard, 1981). OCT's range of roles portends the breadth of physiological responses it modifies, and its effects often vary spatially and temporally throughout the nervous system and responsive tissues. Peripherally, OCT acts as a neuromodulator, often increasing the excitability of neurons innervating the flight muscles and most sensory structures. Notably, OCT also acts within the central nervous system (CNS) and affects arousal, motivation, learning, sensory habituation, thermoregulation, social behaviors, and hygienic behaviors

(Armstrong and Robertson, 2006; Farooqui, 2012; Roeder, 2005). For instance, OCT modulates the response of two locust species (*Locusta migratoria* [Orthoptera: Acrididae] and *Schistocerca gregaria* [Orthoptera: Acrididae]) experiencing intraspecific crowding and induces a shift from developing into a solitary morph into a gregarious morph (Morton and Evans, 1983; Verlinden et al., 2010). OCT concentrations spike in the hemolymph of crickets (*Gryllus texensis* [Orthoptera: Gryllidae]) following discrete exposure to mock predators and associated fleeing behavior (Adamo et al., 2013; Adamo and Baker, 2011). Frequent exposure to a brief blow of air directed at a cricket's cerci also leads to increased OCT concentrations in its hemolymph, although direct exposure to a mantid predator does not induce an OCT increase (Adamo and McKee, 2017). Non-stressed crickets injected with OCT exhibit increased cover-searching and evasive behaviors compared to those experiencing sham injections without OCT (Adamo and McKee, 2017). Overall, OCT is involved in myriad responses to environmental cues, and stressors represent a category of cues that often illicit a strong release of OCT.

Following the release of OCT from octopaminergic dorsal and ventral unpaired median neurons situated near the subesophageal, thoracic, and abdominal ganglia of the CNS, OCT binding to receptors on the neurosecretory corpus cardiacum causes stored AKH to be released into the hemolymph (Kodrík et al., 2015). The release of AKH is conceptually analogous to the release of cortisol from the adrenal gland in the vertebrate HPA axis (Adamo, 2017). AKH has long been known to mobilize lipid reserves from the insect fat body to provide energy to flight muscles during periods of prolonged flight (David et al., 1985; Luo et al., 2014; Ziegler et al., 2011). AKH is also known to act as a potent antioxidant when circulating in the insect's hemolymph (Bednářová et al., 2013; Kodrík and Socha, 2005). Bednářová et al. (2013) find that circulating AKH and protein carbonyl levels (i.e. an indicator of damage from oxidative stress) increase in the firebug *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae) when injected with hydrogen peroxide. Co-injecting AKH and hydrogen peroxide reduced protein carbonyl levels and increased survivorship to control levels, possibly indicating AKHs function as an antioxidant or its role in stimulating the function of other antioxidants (Bednářová et al., 2013). AKH also appears to interact with OCT because octopaminergic neurons throughout the central and peripheral nervous systems are equipped with membrane-bound AKH receptors that allow a neuromodulatory response to hemolymph concentrations of AKH (Adamo, 2017; Farooqui, 2012; Wicher, 2005).

To identify where and when OCT and AKH receptors are acting during a stress response, one must first understand how each receptor functions. Membrane-bound OCT receptors are classified into two main types based on sequence similarity to vertebrate adrenergic receptors: 1) alpha-adrenergic-like receptors (OCTα-R), and 2) beta-adrenergic-like receptors (OCTβ-R; Evans and Maqueira, 2005; Farooqui, 2012). OCTβ-Rs are further divided into three sub-types (Evans and Maqueira, 2005; Farooqui, 2012; Roeder, 2005). Both OCTα-Rs and OCTβ-Rs are activated upon binding with OCT and initiate two non-mutually exclusive, tissue-specific intracellular secondary signaling pathways: 1) cyclic adenosine mono-phosphate (cAMP)/protein kinase A (PKA), and 2) intracellular calcium ($Ca^{2+}$)/inositol-1,4,5-triphosphate ($IP_3$)/diacylglycerol/PKC (Farooqui, 2007; Roeder, 2005). For instance, upon binding with OCT, OCTα-Rs stimulate the concentration-dependent activity of both the intracellular $Ca^{2+}$ and cAMP signaling cascades (Huang et al., 2012; Roeder, 2005). In the $Ca^{2+}$ signaling cascade, for instance, G-protein signaling first activates the phospholipase C-enabled hydrolysis of phosphatidylinositol 4,5-biphosphate into $IP_3$ and diacylglycerol. $IP_3$ then acts on the endoplasmic reticulum to open $Ca^{2+}$ ion channels and causes an influx of $Ca^{2+}$ into the cytoplasm. In the final step of the pathway, diacylglycerol and $Ca^{2+}$ activate protein kinase C (PKC), a family of intracellular signaling proteins common to all eukaryotic cells that functions in phosphorylating numerous proteins, including transcription factors, ultimately leading to transcriptional regulation of stress-associated genes (Altman and Kong, 2016). On the other hand, OCTβ-R activation induces adenylyl cyclase and the production of intracellular cAMP and PKA (Balfanz et al., 2005; Bischof and Enan, 2004; Grohmann et al., 2003; Han et al., 1998). These OCT-induced secondary messenger systems are known to act in a variety of contexts including learning (Schwaerzel et al. 2003), hyperglycaemia (Fields and Woodring, 1991), and phagocytosis (Baines and Downer, 1994; Table 2).

Although numerous insect-specific PKC isoforms exist and their expression is often cell-type- and function-specific (Shieh et al., 2002), recent studies have begun to describe which PKCs function in specific organs within insects. For instance, PKCα is known to specifically act in a $Ca^{2+}$-dependent manner to phosphorylate histones and other transcription regulators, such as methyl DNA-binding proteins, in the *Bombyx mori* brain (Uno et al., 2006). Another PKC subtype known as atypical PKC is necessary for long-term memory maintenance in mouse hippocampal neurons (Jalil et al., 2015) and its activity can rescue *rudish*-mutant *Drosophila* (Diptera: Drosophilidae) from associated memory defects (Shieh et al., 2002), suggesting that PKC-signaling may be important for learning in predator-induced responses. When trying to understand specific cascades of the insect predator-stress response, it is useful to be able to predict which specific PKC isoform is expected to be activated in the tissue and experimental context under study because PKCs are primary post-transcriptional modifiers with specific isoforms tied to discrete roles in transcriptional modification that are restricted to specific tissues.

Although some of the core cellular stress-induced secondary messengers that exist in insects are well-known, no clear pattern is discernible regarding the identification of tissues and/or taxa that may utilize the cAMP or $Ca^{2+}$ pathway preferentially over the other (Table 2). To efficiently target these pathways for study or manipulation, for instance via gene editing or targeted pharmacology, and to validate that such manipulations have an influence on whole-organism performance, it is key to identify which secondary messaging pathway(s) is acting in the context under study. With few prior sub-organismal studies of insect secondary messenger signaling systems in the context of predator stress, there is a critical knowledge gap likely due to the cost-prohibitive molecular assays and expertise required. To overcome these limitations, we recommend that future attempts to discern which insect tissues respond to stressful conditions, particularly in the context of predation risk, should simultaneously monitor cAMP and $Ca^{2+}$ ion levels in discrete tissues sampled at multiple time intervals before, during, and following stressor exposure to disentangle these cell-signaling responses so their respective roles may be characterized. Furthermore, we suggest that researchers assess the gene expression, protein localization, and activity of the two octopamine receptor sub-types and PKC isoforms within the context of predator-induced stress to elucidate the responsible molecular mechanisms. The highly conserved nature of the cAMP and $Ca^{2+}$ signaling pathways in stress responses suggests that the biochemical and cellular mechanisms induced by insect predator stress mirror those more commonly studied in vertebrates and may comprise an evolutionarily conserved, 'generalized' component of the stress response (Fig. 1). Nevertheless, it is critical to know where, when, and how both hormonal and cell-signaling pathways are tied to specific stress-induced responses throughout the insect body, and this knowledge is currently sorely lacking.

## An overview of stress-responsive insect neurohormones

Although the OCT and AKH-signaling systems are traditionally considered simple relative to vertebrate stress-response pathways, the diversity of other hormones, neuromodulators, and downstream physiological effectors induced by the insect stress response implies a high degree of complexity. OCT and AKH exhibit a variety of functions, including the immediate release of neurotransmitters, the induction of extra- and intra-cellular signaling, and the prolonged, often neurally-modulated production and release of other hormones from the paired neurosecretory glands (corpora cardiaca and corpora allata). Even et al. (2012) review the diverse molecular, physiological, and behavioral stress-induced responses that have been reported in honey bees. Honey bee brains exhibit changes in OCT, dopamine, and serotonin concentrations that are correlated with physical stress, age, diel period, seasonality, and can vary among source colonies (Harris and Woodring, 1992). In fruit flies and honey bees, AKH modulates the physiological stress responses to temperature and oxidative damage by initiating intracellular signaling cascades affecting the transcription of antioxidants (Bednářová et al., 2015) and protein chaperones, including heat shock proteins (Elekonich, 2009; Hranitz et al., 2010; Voth and Jakob, 2017). Other signaling hormones that pleiotropically induce physiological responses to stress in insects include juvenile hormone (Chang and Hsu, 2004), a putative cortico-releasing hormone-binding protein (Liu et al. 2011), diuretic hormone-I (Coast, 2006), corazonin (Boerjan et al., 2010; Veenstra, 2009), allatostatin-A (Veenstra, 2009), glucagon-like peptides (Kodrík et al., 2015), and insulin-like peptides (Corona et al., 2007). Glucagon-like peptides and corazonin are both implicated in the activation of antioxidant defenses during periods of oxidative stress in insects, though each is also implicated in broad physiological processes including metabolism and ecdysis, respectively (Kodrík et al., 2015). The potential role of ecdysteroids in the adult stress response is especially of note. In the past 20 years, ecdysteroids, mainly 20-hydroxyecdysone, have been observed to be a primary molecular response to changes in the environment, including nutrient shortage (Terashima et al., 2005), aggressive social interaction (Ishimoto and Kitamoto, 2011), and thermal stress

(Hirashima et al., 2000) in *Drosophila.* Indeed, ecdysteroids and the majority of hormones discussed here act pleiotropically, stimulating many different physiological responses. Notably, the peptide corazonin was originally implicated in mediating cardiac rhythms in cockroaches (Veenstra, 1989), but researchers have since reported its function in locust gregarization, melanization, diel rhythms, and lipid metabolism following nutrient and thermal stress (Boerjan et al., 2010). In fact, the amino acid sequence of the corazonin family of peptides is remarkably similar to that of the AKH family, a pattern that suggests a possible ancestral function of corazonin in priming diel and seasonal stress-responsive physiological systems (Boerjan et al., 2010).

Adamo (2017) proposed a model to explain how a subset of stress-related signaling molecules and gene products could be co-opted from other physiological systems to promote homeostasis during periods of stressor exposure. Adamo (2017) provides evidence that there is substantial overlap in the molecular components induced by predator stress and the molecular components induced by immune challenge. She particularly points out that both predator-stress and immune challenge use biogenic amines (e.g. OCT) and small neuropeptides (e.g. AKH) released from neurosecretory glands into the hemolymph to enhance organismal performance while promoting overall physiological homeostasis. The overlap in components likely accounts for the well-documented trade-off between stress and immune functions through the sharing of scarce molecular resources vital to both responses. One particularly well-studied molecule hypothesized to drive this stress/immune trade-off in insects is apolipophorin III (ApoLpIII), a lipoprotein monomer that is well-known for its role in pathogen recognition pathways (Niere et al., 2001). Following stressor-activated secondary signaling cascades, ApoLpIII in the hemolymph undergoes a conformational change and binds with the apolipophorin I and II complex (Adamo et al., 2008; Adamo, 2017). This conformational change allows diacylglycerol to bind to the complex, forming low density lipophorin, which then transports diacylglycerol from fat body lipid stores through the hemolymph to targeted organs to provide metabolic fuel (Weers and Ryan, 2006; Fig. 2). Illustrating the importance of ApoLpIII, Adamo et al. (2008) find that injecting flight-stressed crickets with AKH reduces free ApoLpIII hemolymph concentrations as it becomes bound with ApoLpI, II, and diacylglycerol, leading to immunosuppression. A similar drop in ApoLpIII concentration might be expected during anti-predator stress responses due to co-option away from its constitutive role in pathogen recognition towards its role in transporting lipids (Adamo, 2017b; Adamo et al., 2008; Noh et al., 2014). To our knowledge, ApoLpI, II, III and diacylglycerol hemolymph concentrations have not been directly measured in insects under predation risk, but this is certainly a rich avenue of future research. The molecules comprising this stress/immune system trade-off in insects are known to act during physiological stress responses related to nutrition (Adamo et al., 2017; Davenport and Evans, 1984; Siegert, 1988), temperature (Taszlow and Wojda, 2015), pathogen infection (Adamo, 2017), and predator exposure (Boonstra, 2013; Hawlena et al., 2011; McPeek et al., 2001; Van Dievel et al., 2016). In fact, the appearance of OCT and AKH receptors on the extracellular membranes of both efferent sensory neurons (Lam et al., 2013; MacDermid and Fullard, 1998) and immune-induced hemocytes further suggest an ancestral signaling relationship between these two systems (Adamo, 2017).

Indeed, animals across phyla can modulate immune function during interactions with predators (Adamo, 2012), implying that the ability to optimize stress and immune responses according to transient needs has been conserved for over 400 million years (Adamo, 2017). The deep evolutionary history of animal stress responses must be acknowledged and integrated into future studies of comparative evolutionary physiology. Such an approach will pave the way for an integrative understanding of how nonlethal, predator-induced stress can scale to affect population demography and fitness in natural and anthropogenic systems. Demonstrating the influence of predators not only on direct mortality, but also the stress physiology and organismal performance of pest insects, could also aid in the development of predator-based approaches to sustainable agriculture. For instance, fifth instar *Manduca sexta* (Lepidoptera: Sphingidae) larvae that were pinched with forceps 8 times over the course of 30 sec repeatedly for three hours exhibited reduced mass gain, delayed development, increased hemolymph octopamine levels, reduced hemolymph glutathione (an antioxidant discussed below) levels, and increased antimicrobial peptide *attacin-1* expression (Adamo et al., 2017). Clearly, frequent exposure to a mock predator can have marked indirect effects on both the physiology of exposed adults and even offspring performance, with promising results for biological control strategies based on the ecology of fear, because *Manduca sexta* is commonly used to model processes in agricultural pest moths, such as *Helicoverpa* or *Spodoptera.* A thorough understanding of predator-induced stress physiology in insects could also help avoid extinctions. Endangered insect species, particularly those displaced by an invasive predator, may experience population declines in excess of what direct mortality predicts. Similarly, greater understanding of the underlying physiology of the ecology of fear may provide insights into the performance of beneficial insects, from pollinators to biological control agents. Studies of insect sensory processing and downstream neurophysiological stress can further be applied to more immediately practical pursuits, including the production of targeted insecticides and developing translational models of human sensory diseases (Albert and Göpfert, 2015; Senthilan et al., 2012; Song et al., 2001) and other neural disorders (Clinchy et al., 2011; Pandey and Nichols, 2011).

## An integrated metabolic-, immune-, and predator-induced stress response in insects

Both acute and chronic predator-induced stressors are prevalent in nature (Boonstra, 2013; Clinchy et al., 2013) and both broadly impact the behavior, physiology, life history, and fitness of many insects (Adamo, 2017; Hawlena et al., 2011; Rodríguez and Greenfield, 2004; Zha et al., 2013). To date, most studies of insect physiology under predation risk have focused on energetics and whole-organism responses, as opposed to studying signaling pathways and their accompanying cellular and biochemical responses. For example, in two damselfly species, *Enallagma aspersum* (Odonata: Coenagrionidae) and *Ischnura verticalis* (Odonata: Coenagrionidae), circulating hemolymph concentrations of glucose and glycogen were unchanged in the presence of predators, though triglycerides were decreased in *Enallagma* but not *Ischnura*, while total protein levels decreased in *Ischnura* but not *Enallagma* (McPeek et al., 2001; Stoks et al., 2005). However, in a study that did investigate sub-organismal molecular responses to predation stress, Slos and Stoks (2008) found that the abundance and activities of cellular effectors, including heat shock protein, Hsp70, and the antioxidant enzyme, catalase, were enhanced under predation risk in *Enallagma cyathigerum,* another damselfly species. These same cellular effectors have previously been found to be involved in many stress responses, from heat to desiccation among others, nominating them to be part of a generalized stress response system in insects (Hermes-Lima and Zenteno-Savín, 2002).

With the knowledge that molecular resources are shared between the generalized stress and immune responses in insects, one might hypothesize that the same molecules observed in nutritionally, oxidatively, or immunologically stressed insects would also play a role in predator-stressed insects. One such molecular response involves glutathione, a major antioxidant found in insects (Clark et al., 2010) that acts through its capacity for neutralizing free radicals and reactive oxygen species. However, high hemolymph concentrations of glutathione also prevent the reactive products of phenoloxidase, a key line of immune defense, from acting as a pathogen suppressor (González-Santoyo and Córdoba-Aguilar, 2012). For example, Stahlschmidt et al. (2015) find that female crickets (*G. texensis*) experimentally infected with a bacterium display a positive correlation in hemolymph glutathione concentration and fecundity. Further, the ratio of glutathione to prophenoloxidase circulating in the hemolymph is unaffected by food or immune challenge, indicating a tight balance between the free radical-producing effect of prophenoloxidase activity and its mitigation by glutathione (Stahlschmidt et al., 2015). By explicitly correlating fast-acting stress signaling cascades, such as octopamine and AKH signaling, with downstream cellular-level effector activity, one can build mechanistic hypotheses regarding specific signaling systems and the discrete sets of responsive effector molecules activated as part of a specialized stress response vs. general stress responses. Kodrík et al. (2015) and Farooqui (2012) review the functions of AKH and OCT signaling in insect stress responses, respectively, and show that both affect tissue-specific enzyme activity and transcriptional regulation by acting through distinct secondary messengers (Table 2). Although great strides have been made in model organisms like *Drosophila* with projects like FlyAtlas (Chintapalli et al., 2007) and ModEncode (Celniker et al., 2009), research is now needed that can discern the specific tissues and cell types expressing one or more of the OCT and/or AKH receptor subtypes and their discrete impacts on tissue and organismal physiology in non-model organisms across different ecological contexts (i.e. acute vs. chronic stressors) to advance a mechanistic understanding of cellular-, transcriptional-, effector-, and organismal-level responses to predation risk, as well as ultimately population- and community-level responses due to the ecology of fear. As one of the few experimental examples spanning these scales from sub-organismal to whole-organism responses while also comparing acute and chronic stressors, crickets (*G. texensis*) experiencing chronic immune challenge every 3 days shift molecular resources towards oxidative stress tolerance relative to immune resistance, whereas immune resistance is highly activated during acute immune challenges (Stahlschmidt et al., 2015). Exposure to acute and chronic predation risk often induces complementary, yet distinct, physiological responses, and pinpointing where, when, and what molecular interactions are involved in each remains a key area of study in the field of insect stress.

## Sensory and taxonomic biases of studies on predator-induced stress across insects

Insects are well-known for their array of sensory mechanisms, including several forms of lineage-specific sensory receptors. For instance, female *Ormia ochracea* [Diptera: Tachinidae] flies use specialized, paired tympanal organs to localize singing *Gryllus* crickets, which they then parasitize (Robert et al., 1992). Each receptor type is correlated with a spectrum of behavioral responses that is induced upon the perception of environmental cues, although multi-modal sensory integration can lead to diverse, non-normative reactions. Notably, *Drosophila* respond with two distinct behavioral repertoires depending on the speed and size of a visual stimulus (Wu et al., 2016). Each behavioral response is represented neurophysiologically by activity in distinct neurons within the same stimulus-encoding layers of the optic lobe (Klapoetke et al., 2017). For example, a looming object, one that is moving towards the subject, activates a distinct lobula columnar and neuron sub-type in the *Drosophila* eye that innervate the giant fiber escape circuit and leads explicitly to a prescribed turning and escape behavior (von Reyn et al., 2017; Wu et al., 2016). These prescribed behavioral regimes and specific underlying neural responses have allowed neuroscientists to map the discrete activation of sensory-processing neuron sub-types sensitive to specific cues, including looming-sensitive neurons, that encode similar behavioral escape responses in all animals studied thus far (Peek and Card, 2016). This field of research reveals the deep homology or convergence of these neural responses to a predator-related visual cue and emphasizes the need for more detailed comparisons between the sensory systems studied, the methods used to activate specific neural sub-types, and the distinct parts of the brain where higher-order processing of these stimuli occurs among diverse evolutionary lineages.

Despite significant technological advancements to describe neural responses to stressors, the methodology employed in studies of molecular stress responses remains somewhat limited. Current molecular assessments of stress in insects, which primarily use *Drosophila melanogaster* as a model, employ whole-body or whole-tissue sample extractions that can bias the resulting observations and their interpretation. Compared to vertebrates, the relatively simple insect nervous system provides an opportunity to compare stress responses in fine-scale studies of predation-related neurophysiology through the use of microdissections, single-cell analytic technologies, optogenetic labeling, and elegant histological techniques. Along with technological advancements in measuring biochemical stress responses, a foundational knowledge of predator and prey contexts is required to understand how nonlethal effects of predation risk influence an organism's physiology. Among these considerations are predator and prey life histories, the sensory modalities involved in predator-prey interactions, and both the acute and chronic neurophysiological responses that are expected to occur. Due to body size constraints on microdissection and the prohibitive amount of tissue required for effective molecular sequencing studies, deconstructing a physiological stress response into sensory perception, lower- and higher-order processing, and local compared to systemic responses is currently limited in non-model insects. Yet, it is only a matter of time before such approaches are translated to novel, non-model species by building on the genetic tools developed for studying neurophysiological stress in *Drosophila*. Many insect predator-prey interactions are studied at the behavioral and organismal levels, yet the stimuli used among different studies are often not directly comparable. For example, mechanically shaking an insect is often assumed to be a proxy for a predation-related cue, though little empirical evidence supports this assumption (Evans et al., 2012). Other studies have relied on more ecologically relevant and quantifiable stimuli, including predator kairomones (Van Dievel et al., 2016; Heads, 1985), auditory exposure to predator sounds (Huang et al., 2003; Minoli et al., 2012; Zha et al., 2013), visual cues (Combes et al., 2012; De La Flor et al., 2017; Kacsoh et al., 2015), or allowing direct interaction between predator and prey by rendering predators harmless (Persons et al., 2001; Schmidt-Entling and Siegenthaler, 2009; Snyder and Wise, 2000). The variety and context-dependence of cues used throughout the literature makes comparisons between studies challenging. Additionally, outcomes of many predator-prey interactions are often context-dependent, with strong influences of resource availability (both for predator and prey), conspecific cues, and transient organismal physiology impacting anti-predator behavior in both vertebrates (Clinchy et al., 2004; Drakeley et al., 2015; Figueira and Lyman, 2007; Katwaroo-Andersen et al., 2016; Oliveira et al., 2017; Pike et al., 2010) and invertebrates (Charalabidis et al., 2017; Dittmann and Schausberger, 2017; Elliott et al., 2017; Jones and Dornhaus, 2011; Laws and Joern, 2015; Stahlschmidt et al., 2014; Stahlschmidt and Adamo, 2015).

Another roadblock to synthetic understanding is that the literature on predator-induced stress in insects is scattered across highly divergent orders (i.e. Orthoptera [Goosey, 1982; Morton and Evans, 1983; Verlinden et al., 2010)], Diptera [Kaufmann et al., 2009; Rahman et al., 2013], Lepidoptera [MacDermid and Fullard, 1998; Pfuhl et al., 2015], and Odonata [McPeek et al., 2001; Slos and Stoks, 2008; Van Dievel et al., 2016]). Over hundreds of millions of years, these taxa have diverged to produce the myriad sensory receptor organs and associated behavioral responses to environmental stimuli for which each clade is now well-known (Lozano-Fernandez et al., 2016;

Misof et al., 2014; Table 1, Fig. 3). In one example, Crespo (2011) reviews the chemosensory structures, relevant neural circuitry, and associated olfaction-induced behavioral responses among a majority of aquatic insect orders and emphasizes the extreme diversity seen in neural structure and organization, even within the life cycle of a single holometabolous species. Crespo (2011) cites the common misassumption that Odonata are solely visual navigators. Although odonates were originally thought to have little, if any, olfactory sense due to their lack of glomerular antennal lobes like those found in Neoptera, odonate nymphs are now recognized to use olfactory kairomones for predator avoidance (Crespo 2011). As a result, Crespo (2011) recommends studying both organismal life history and the functional arrangement of sensory neuron connections among specific neuropils to accurately assess neurophysiological responses from the moment of cue perception to the activity of downstream physiological effectors.

In the context of predation risk, the modalities of insect visual and olfactory neurophysiology have been relatively well-studied (Borst, 2009; Crespo, 2011; Gabbiani et al., 1999; Hatsopoulos et al., 1995). Other sensory modalities in insects, such as hearing, are only now becoming recognized for their influence on prey physiological responses to predation, despite a rich neuroethological history (Hoy et al., 1989; Hoy and Robert, 1996; Ratcliffe et al., 2011). These advances in insect sensory ecology represent exciting opportunities to compare neural structure, organization, complexity, and function when individuals are exposed to stimuli with different sensory modalities (e.g. auditory vs. visual), both within a single species and across diverse evolutionary lineages. Ancestral proprioceptors and chordotonal receptors have been co-opted as tympanal organs in many insect orders, and have independently evolved in at least 20 locations throughout the basic insect body plan (Göpfert and Hennig, 2016). Though neurophysiological data on auditory neurons and processing in insects is limited, a few studies have described the neural architecture of auditory perception in noctuid moths (Lepidoptera: Noctuidae; MacDermid and Fullard, 1998; Pfuhl et al., 2015; Roeder, 1966a; ter Hofstede et al., 2011). The afferent auditory neurons of the noctuid tympanal organ extend into the fused meso- and metathoracic (pterothoracic) ganglia before a single ascending interneuron transmits a signal to the ventrolateral protocerebrum (Pfuhl et al., 2015; ter Hofstede et al., 2011). Another notable auditory receptor is the near-field responsive Johnston's organ that is situated on the insect antenna and is innervated by afferent neurons extending first to the antennal lobes before reaching higher-level processing centers (Caldwell and Eberl, 2002). Although its function in predator detection is debated, the Johnston organ is intimately involved in mating and social interaction in many insects and thus may also provide sensory information pertaining to potentially stressful conditions, such as antagonistic social interactions. To parse the intermediary processes that may modulate responses by particular auditory neural pathways, recent work has established that long-term exposure to bat calls over the course of 8 hours induces several transcripts encoding components of G-protein-coupled receptor activation, $Ca^{2+}$ ion release, a heat shock protein, and mitochondrial metabolism that are differentially regulated in the whole brain tissues of male *Spodoptera frugiperda* (Lepidoptera: Noctuidae; Cinel and Taylor, 2019). Continued advances in molecular sequencing and the characterization of non-model insects will likely bring many understudied insect predator-prey interactions to the forefront of sensory and stress physiology research.

Although some types of stress responses in insects have been well-investigated (e.g., thermal stress as reviewed by King and MacRae, 2015; Overgaard and MacMillan, 2017), in most cases, the discrete sensory processing, signaling, and regulatory events associated with transcriptional responses to predator-induced stress are not yet known, making it difficult to accurately compare stress responses without bias. Variation in physiological stress responses between divergent arthropod classes is perhaps unsurprising, yet even closely related insects appear to utilize functionally distinct physiological pathways to promote homeostasis in the face of stressors (Bednářová et al., 2013; Gäde et al., 2008; Gog et al., 2014; Kaufmann et al., 2009; Salim et al., 2017; Zhang et al., 2008). For example, predator cues have been shown to induce oxidative stress in damselflies, but the antioxidant effectors associated with these responses in stressed insects can be distinct across sensory contexts. Specifically, Janssens and Stoks (2013) found that concentrations of the antioxidant enzyme superoxide dismutase, but not the antioxidant enzyme catalase, decreased in the damselfly *Enallagma cyathigerum* (Odonata: Coenagrionidae) larvae following exposure to just a larger damselfly predator. In contrast, Slos and Stoks (2008) reported in *E. cyathigerum* a decrease in catalase concentrations, but not superoxide dismutase, following simultaneous visual and chemosensory exposure to cannibalistic conspecifics, conspecific alarm pheromones, and a fish predator simultaneously. This example of a single species altering the concentration of complementary antioxidant effectors in opposite ways under different predator stress contexts drives home the point that responses to ostensibly similar stressors may have distinct perceptual and molecular bases.

Like many other animals, insects likely display context-dependent physiological responses that might be driven, for instance, through genetic heritable variation, epigenetic inheritance, or early life exposure (Adamo et al., 2013; Bell and Sih, 2007; Kain and McCoy, 2016; Mcghee et al., 2012; Watts et al., 2014). The freshwater snail *Physa acuta* (Heterobranchia: Physidae) increases anti-predator hiding behavior and decreases fecundity in response to predation risk imposed by *Procambarus* crayfish (Decapoda: Cambaridae). Yet, these responses occur in some individuals and not others, particularly under intermediate and stochastic levels of predator cue exposure (Kain and McCoy, 2016). These interactions exhibit personality-driven behavioral and physiological differences observed during similar predator-prey and antagonistic social interactions in animals from carpenter ants (d'Ettorre et al., 2017; Hawlena et al., 2011; Rose et al., 2017) to stickleback fish (Blake and Gabor, 2014; Fürtbauer et al., 2015; Mcghee et al., 2012). However, there is still a lack of rigorous experimental evidence that demonstrates inter-individual variation in response to predation while accounting for sensory and environmental context in insects.

## A standardized approach to studying predator-induced stress in insects

An integrative approach that incorporates context- and taxon-dependent differences in stress responses is needed to properly characterize spatiotemporal, sub-organismal molecular responses to predation-related stressors in insects. A framework describing the sequence of events induced by predator cue perception is helpful for conceptualizing the molecular mechanisms associated with predator-induced stress and to inform proper timing of sampling for making inferences about particular components of predator exposure responses. The sequence of events that occur upon exposure to a predator-cue includes cue detection, signal transduction, systemic biogenic amine and neuropeptide release, transcriptional regulation, post-transcriptional modifications, and effector activity, though the order of these processes is not necessarily linear (Fig. 4).

Stemming from systems biology, a physiological regulatory network perspective examines the interrelatedness of individual physiological components and the numerous molecular pathways that include each component as part of a larger organismal homeostatic system (Adamo, 2017; Cohen et al., 2012). A physiological regulatory network approach involves studying how physiological components interact at one level of a network and how those interactions influence physiological components at lower and higher levels of organization. For instance, a physiological regulatory network approach might include the simultaneous measurement of stress and immune responses as part of a larger homeostatic defense system (Adamo, 2017; Cohen et al., 2012). Utilizing this physiological regulatory network perspective can help demystify seemingly incongruent patterns in a given system's response to a stressor by looking for possible interactions with other integrated physiological systems. In the context of this review, a physiological regulatory network perspective could represent a mechanistic understanding of how the stress, immunity, and metabolic systems, for instance, influence each other in response to predation stress. This shift towards a physiological regulatory network perspective has contributed to several discoveries of tightly integrated biochemical resource allocation pathways, including the aforementioned immune/predator-induced stress trade-off (Adamo, 2017a; Adamo et al., 2017) and immune resistance/oxidative damage trade-off (Stahlschmidt et al., 2015). Specifically, Adamo (2017) uses a physiological regulatory network perspective to frame trade-offs between the allocation of ApoLpIII to stress and immune functions and explain how these integrated systems work in concert to prime immune function in an animal's current ecological context by sharing and borrowing molecular effectors between their respective response pathways. Adamo (2017) cites the increase in antimicrobial protein expression many insects display following mechanical stress, but not infection, as a possible reconfiguration of the immune system to compensate for changes elsewhere in the network, such as the co-option of ApoLpIII from its role in immunity towards lipid transport. Additionally, studies of prey physiology should confirm that a whole-organismal response, i.e. a change in fitness or fitness-related performance parameters, tied to the sub-organismal physiological parameters under study indeed occurs. Too often, sub-organismal molecular and cellular studies are separated from important organismal performance parameters.

Despite recent advances in understanding how the stress and immune systems integrate within the context of a generalized stress response, a broad, network-level approach to measuring OCT, AKH, and associated transcriptional and regulatory activities following exposure to predator stimuli is still lacking (see Adamo, 2017). In insects, sensory modalities are thought to be integrated through the transmission of peripheral stimuli to processing centers of the proto-, deuto-, and trito-cerebral segments of the brain (Farris, 2005). However, the segmented, distributed ganglia comprising the insect CNS apart from the brain likely contain many other non-cerebral, multi-modal processing centers. Determining the discrete location of sensory modality processing in the CNS of specific insect clades would provide additional evidence for pinpointing neurosecretory tissues to which molecular and neural manipulation experiments can be effectively applied. The discrete localization of nervous transmission within the insect CNS also helps ensure that the genes and proteins identified as functionally relevant are indeed related to the stressor under investigation (Evans, 2015).

Once a researcher has identified a tractable predator-prey system, we recommend the use of a general 5-step protocol to holistically assess the effects of predation-related stress on insect prey (Fig. 5). First, one must **establish the ecological relevance** of the predator/cue exposure used and explicitly calibrate exposure to detect effects of stimulus over- and under-exposure relative to the natural stimulus frequency. One should consider the distinction between acute and chronic exposure to a particular predator cue and attempt to emulate one or the other based on the question of interest. For instance, seemingly small differences in a cue can have dramatic influences on prey physiology and behavior. Studies of eared moths and their responses to ultrasound provide a valuable example of how the use of a proxy cue, one similar yet usually of questionable ecological relevance, can have a strong influence on experimental outcome. Specifically, many studies have used synthetic ultrasonic signals to recreate a bat call-like stimulus (Svensson et al., 2003; Zha et al., 2013), but these stimuli often lack various qualities of the real stimuli they are mimicking and can produce quite different neural responses in the receiving individual. Until a comparative study of the influence of both bat-like ultrasound and recorded bat calls on moth behavior and physiology is conducted, the ecological relevance of these studies remain in question. Next, one should **establish the effect size** of predator/cue exposure on organismal behavior, life history, physiology, and ultimately fitness. For instance, by simultaneously measuring a prey species' baseline or control response, the response to predator exposure, and the response to a benign cue, one can then parse the effect of predator exposure *per se* from any effect that may simply be due to sensory stimulation alone. Once the effect size has been established, and is hopefully substantial, the next step is to **identify complementary functional hypotheses** based on the neurophysiology of cue and/or risk perception (i.e. sensory modality, transient versus persistent cues, length and frequency of exposure), make an assessment of biochemical and cellular signaling molecules expected to be involved in the neurophysiology of cue perception (i.e. OCT/AKH hemolymph titers, ligand receptors, cAMP, $Ca^{2+}$, PKA, PKB, PKC, $IP_3$, FoxO), and make an assessment of stress-activated physiological effector molecules known to act in the same or closely related taxa (i.e. neuromodulators, diacylglycerol, HSPs, antioxidants, ApoLpI, II, III). Previous studies and published hypothesized molecular pathways provide a rich source of information from which specific predictions of the molecules involved during a given prey insect's response to a predator can be constructed. Building on the knowledge accrued during steps 1 – 3 (above), one can **leverage informational and resource needs** with available technologies, resources, and expertise. Specifically, we recommend considering data interpretability, with an emphasis on the need for RNA versus protein-level evidence, sample preparation and analysis costs, and instrumental access. Specialized kits, instrumentation, and expertise are often required to conduct molecular assays, with standardized RNA and DNA extraction and sample preparation kits readily available compared to the highly customized nature of mass spectrometry and high-performance liquid chromatography. Finally, access to specialized analytical tools should also be considered, such as utilizing high-performance computing resources, scripting languages, and RNA-Seq or proteomic databases and software. Finally, one should **plan for further validation** of mechanisms nominated with genetic loss-of-function/gain-of-function, field-based, behavioral, neurophysiological, and other experimental approaches. Once a well-defined correlation between biochemical, transcriptional, or physiological responses and predation risk has been established, further experimental validation must be used to demonstrate the influence of predation risk on the organismal phenotype, or even population and community demographics, within an ecologically relevant framework. Studies demonstrating the heritability of predator-induced stress responses, loss-of-function studies using nerve cell ablation, or genetic knock-out techniques that show a clear lack of response, and comparative studies of predator stress-induced life history changes operating in natural conditions are among the most promising avenues for future research.

## Conclusions

Many molecular and physiological responses to stressors have been documented from insects during and after predator interactions. A few molecules, namely AKH, OCT, and several antioxidants, are shared between metabolic-, immune-, and predator-induced stress responses. We suggest that the octopamine-adipokinetic hormone (OAH) stress response in insects is analogous to the well-described vertebrate HPA axis. We emphasize the need to account for taxon- and sensory-specific differences among study organisms and the methodologies used when comparing results. Additional research is needed to effectively characterize neurohormonal, secondary messenger, transcriptional, and effector molecule pathways, to describe how these pathways influence stress physiology over the short and long terms, and to explore the use of functional genomic methods for pinpointing the role of specific genes and their products within a given species' physiological and behavioral stress response. We suggest that some particularly fruitful research may be to: (1) parse the roles of specific OCT receptor sub-types in context-specific stress responses, (2) determine the specific transcriptional modifications made and effector molecules produced across an insect's tissues during and after interactions with predators, with special care spent to select time points appropriate to the processes involved, and (3) establish hypothesized physiological regulatory networks for predator-induced stress responses in insects specifically. Extensions of knowing which OCT-receptor sub-type, if any, is responsible for eliciting predator-induced stress responses in insects may allow the targeted knockdown of predator-responsive physiological pathways for use in future pest management applications. Next, knowing where and when stress-induced transcriptional modifications are being made and effector molecules are active during an exposure to a predator can help build a mechanistic understanding of how tissues and molecules interact throughout the entire organism. Finally, establishing a hypothesized physiological regulatory network for predator-induced stress in insects would provide many benefits, including guidance for understanding stress physiological data from wild insect populations of conservation concern or beneficial insects, from pollinators to biological-control agents, that may be targeted by native or non-native predators.

Advances in transcriptome sequencing of non-model insects has made it feasible to attain tissue-, or even single-cell-, specific descriptions of gene expression before, during, and after an interaction with a predator. By correlating anti-predator behavioral regimes with stress-induced neurohormonal signaling, tissue-specific gene expression, and downstream effector activity, one may be able to assess the diversity of physiological responses observed in stressed insects. We suggest that the evasive flight maneuvers and production of jamming ultrasound in response to bat calls reported in several ultrasound-sensitive lepidopteran clades are examples of easily inducible behavioral regimes that can be paired with tissue-specific and time-series molecular techniques within different stress contexts (Barber and Kawahara, 2013; Corcoran et al., 2009; Kawahara and Barber, 2015; Roeder, 1966; Werner, 1981; Yack and Fullard, 2000). These suggested investigations and others will allow the field to build a holistic understanding of how individual components of physiological regulatory networks interact to enable appropriate physiological changes to stressors that can affect prey species from the sub-cellular to the organismal scales and beyond, with repercussions for population and even community-level processes (Fig. 6).

A replicable and reliable approach to measuring stress-induced molecular responses is needed to better understand the physiology of prey insects. First, studies of predator-induced stress should incorporate a wide range of molecular responses when describing or hypothesizing about physiological and biochemical pathways to allow us to uncover lineage- and sensory-specific responses. For example, by explicitly measuring neurohormone levels, receptor protein activity, secondary messenger activity, transcriptional regulation, and effector protein/molecule activity within the context of predator exposure, one can build a well-defined network of interacting molecular components. Future investigations describing the interactions of metabolic-, immune-, and predator-related stressors on the molecular pathways we review here will eventually yield an intricate description of how these integrated systems function across ecological contexts, ranging from starvation to predator pressure. Second, it is crucial that comparative investigations of neurophysiological stress responses examine similarities not only between phyla (Adamo, 2017; Bednářová et al., 2013) but also between closely related species (Gade et al., 2008; Lam et al., 2013; Papaefthimiou and Theophilidis, 2011). Such an approach will enable a holistic understanding of the similarities and differences between taxon-specific, sensory-specific, and universal stress responses. Novel technologies, such as transcriptome sequencing from the level of single cells to organs, proteome-wide mass spectrometry, and gene editing tools provide the means necessary to measure and perturb entire physiological regulatory networks simultaneously. Yet these approaches also have limitations. For instance, the indirect relationship between mRNA expression, alternative splicing, posttranslational protein modifications, and protein activity (Evans, 2015) continues to limit the interpretation of transcriptomic measurements. Technical limitations will surely diminish as robust genomic, transcriptomic, and proteomic resources continue to develop that allow for the inexpensive, practical identification and quantitative measurement of active proteins, peptides, and their up- and down-stream derivatives from diverse insect taxa, tissues, developmental stages, and predation contexts.

The best-practice guidelines provided here can help nominate molecular constituents of predator-induced stress in insects to explore how these molecules and pathways are evolutionarily related. Specifically, we emphasize the importance of ecological context in designing stress-response assays as well as identification of lineage-specific and shared molecular pathways involved in physiological acclimation to predator presence and the application of these findings towards the understanding of human disease, ecophysiological research, conservation, and sustainable pest management.

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## Tables and Figures

Table 1. List of studies investigating the behavioral, physiological, and molecular responses of insects to predation-related stress, with emphasis on the type of predator-prey interaction tested, sensory modalities involved, responses measured, and reported molecular pathways and genes involved.

Summary of Molecular, Physiological, and Behavioral Responses of Insects to Predator-Induced Stress

| Order | Species studied | Predator or cue used | Sense(s) involved | Physiological or other response(s) | Hypothesized or known pathway(s) and gene(s) involved | Reference(s) |
|---|---|---|---|---|---|---|
| Coleoptera | *Leptinotarsa decemlineata* | *Podisus maculiventris* | olfactory, tactile, visual | reduced feeding | - | Hermann and Thaler (2014) |
| Coleoptera | *Cicindela marutha* | simulated bat ultrasound | auditory | behavioral change and induction of auditory defenses | - | Yager and Spangler (1997) |
| Diptera, Trichoptera | *Chironomus riparius, Sericostoma vittatum* | *Salmo trutta* | olfactory | impaired growth and delayed adult emergence | - | Pestana et al. (2009) |
| Diptera | *Drosophila melanogaster* | *Leptopilina heterotoma, Plexippus paykulli, Phyllovates chlorophaea,* mock predator | tactile, visual | reduced oviposition, increased exploratory behavior, transmission of responses to naïve flies | *drice, Dcp-1, Orb2, Adf1, dnc, rut, FMR1, amn, wg, EWG* | Kacsoh et al. (2015); de la Flor et al. (2017) |
| Hemiptera | *Acyrthosiphon pisum* | (E)-β-farnesene (alarm pheromone) | olfactory | altered feeding locations | - | Keiser and Mondor (2013) |
| Hymenoptera | *Apis mellifera* | shaking, alarm pheromone exposure, pinching leg, electric shock | tactile, olfactory, visual | increased aggression, elevated levels of biogenic amines and antioxidants | OCT- and AKH-induced regulation of downstream gene expression | Evans et al. (2012) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Lepidoptera | *Amphipyra pyramidoides, Caenurgina erechtea, Feltia jaculifera, Phlogophora periculosa, Lymantria dispar, Ennomos magnaria* | simulated bat ultrasound | auditory | reduced flight activity | - | Fullard et al. (2003) |
| Lepidoptera | *Pygarctia roseicapitis, Cisthene martini* | direct exposure to bats | auditory, tactile, visual | behavioral change, induction of auditory defenses, increase in survival | - | Dowdy and Conner (2016) |
| Lepidoptera | *Ostrinia nubilalis, Pseudaletia unipuncta* | simulated bat ultrasound | auditory | reduced and interrupted mating behaviors | - | Acharya and McNeil (1998) |
| Lepidoptera | *Helicoverpa armigera, Plodia interpunctella* | simulated bat ultrasound | auditory | increase and decrease in fecundity, respectively | - | Zha et al. (2013); Huang and Subramanyam (2004) |
| Lepidoptera | *Bertholdia trigona, Cycnia tenera* | simulated bat ultrasound and direct exposure to bats | auditory, tactile, visual | behavioral change and induction of auditory defenses | - | Corcoran et al. (2013); Ratcliffe et al. (2011) |
| Lepidoptera | several sphingid spp. | simulated bat ultrasound and direct exposure to bats | auditory, tactile, visual | behavioral change and induction of auditory defenses | - | Kawahara and Barber (2015) |
| Lepidoptera | *Galleria mellonella* | - | tactile; shaken in hands | increased hemocyte density, elevated *galiomicin* and *inducible metalloproteinase inhibitor* transcription, elevated apolipophorin, arylphorin, and prophenoloxidase expression | immune priming | Brown et al. (2014); Mowlds et al. (2008) |
| Mantodea | several hymenopid, mantid and empusid spp. | direct exposure to bats | auditory, tactile, visual | behavioral change and induction of auditory defenses | - | Triblehorn and Yager (2001) |

|  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
| Odonata | *Enallagma cyathigerum, E. aspersum, Ischnura verticalis* | *Anax imperator, Gasterosteus aculeatus* | olfactory, tactile, visual, other* | reduced growth rate, reduced feeding, increase metabolism, reduced nitrogen-rich protein content, reduced carbon-rich fat and sugar content (whole body), increased antioxidant and reactive oxygen species activity | glyconeogenesis, reduction in catalase levels | Janssens and Stoks (2013); McPeek et al. (2001); Slos and Stoks (2008); Stoks et al. (2005); Van Dievel et al. (2016) |
| Odonata | *Coenagrion puella, Ischnura elegans* | *Gasterosteus aculeatus, Pungitius pungitius* | olfactory, tactile, visual, other* | reduced activity | - | Convey (1988) |
| Odonata | *Enallagma ebrium, Ischnura verticalis* | *Lepomis gibbosus* | olfactory, tactile, visual, other* | reduced feeding | - | Baker and Dixon (1986) |
| Odonata | *Leucorrhinia intacta* | *Anax junius, Lepomis macrochirus* | olfactory, tactile, visual, other* | increase in immune encapsulation, induction of abdominal spines | immune priming | Duong and McCauley (2016); McCauley et al. (2011) |
| Orthoptera | *Gryllus texensis* | perception of predation risk, exposure to mock predator | auditory, olfactory, tactile, visual, other* | altered oviposition site selection, increased OCT hemolymph levels, decreased feeding and metabolism | OCT-mediated metabolic responses | Adamo and Baker (2011); Stahlschmidt and Adamo (2013) |
| Orthoptera | *Melanoplus femurrubrum* | *Hogna rabida, Pisaurina brevipes, P. mira, Phidippus rimator, Tibellus maritimus, T. oblongus* | auditory, olfactory, tactile, visual, other* | feeding, activity, and habitat use | - | Miller et al. (2014); Hawlena et al. (2011) |
| Orthoptera | *Locusta migratoria, Schistocerca gregaria* | induction of flight activity, handling | tactile, visual | lipid mobilization | AKH-mediated signaling and induction of glycogen and DAG transport in hemolymph | Orchard et al. (1981); Van der Horst and Rodenburg (2010) |

Abbreviations: *Adh1* (*Adh transcription factor 1*); AKH (adipokinetic hormones); *amn* (*amnion associated transmembrane protein*); DAG (diacylglycerol); *Dcp-1* (*death caspase-1*); *drice* (*death related ICE-like caspase*); *dnc* (*dunce*); *EWG* (*erect wing*); *FMR1* (*fragile X mental retardation 1*); OCT (octopamine); *Orb2* (*translational regulator Orb2*); *rut* (*rutabaga*); *wg* (*wingless)*
Note: Studies in which prey were exposed directly to predators are marked with a '*'.

Table 2. List of studies assessing intracellular secondary messenger activity following octopamine exposure/binding in insects and other invertebrates, including details on the species studied, the tissue or approach utilized, any physiological response observed, and whether the cAMP/PKA or $Ca^{2+}$/PKC signaling pathways were induced (X), not affected (-), or not assessed.

| Induced secondary messenger pathway | | Species | Order | Tissue and/ or approach | Physiological and/or behavioral response(s) | Reference(s) |
|---|---|---|---|---|---|---|
| cAMP/PKA | calcium/PKC | | | | | |
| X | X | *Periplaneta americana* | Blattodea | hemolymph; cloned in COS-7 and HEK-293 cells | - | Bischof and Enan (2004); Gole et al. (1987) |
| not assessed | X | *Periplaneta americana* | Blattodea | activity of calcium-signaling inferred by proxy through $IP_3$ activity in cultured *P. americana* cells | increased phagocytois | Baines and Downer (1994) |
| X | not assessed | *Periplaneta americana* | Blattodea | thoracic ganglia, *in vivo* and intact preparations | - | Nathanson and Greengard (1973) |
| X | X | *Drosophila melanogaster* | Diptera | *Drosophila melanogaster*-cloned receptors expressed in cultured HEK-293 cells | - | Balfanz et al. (2005) |
| X | X | *Drosophila melanogaster* | Diptera | *Drosophila melanogaster*-cloned receptors expressed in cultured Drosophila S2, CHO, and HEK-293 cells | - | Han et al. (1998); Robb et al. (1994) |
| X | - | *Drosophila melanogaster* | Diptera | *Drosophila melanogaster*-cloned receptors from head or body expressed in cultured CHO-K1 cells | - | Maqueira et al. (2005) |
| X | not assessed | *Drosophila melanogaster* | Diptera | brain (mushroom body Kenyon cells) | appetitive learning | Schwaerzel et al. (2003) |
| X | X | *Apis mellifera* | Hymenoptera | expression of AmOA1 receptor in cultured HEK-293 cells | - | Grohmann et al. (2003); Farooqui 2007, (2012) |

| | | | | | | |
|---|---|---|---|---|---|---|
| X | not assessed | *Apis mellifera* | Hymenoptera | whole-brain homogenate | - | Balfanz et al. (2014) |
| not assessed | X | *Apis mellifera* | Hymenoptera | ectopic exposure of whole-brain tissue to octopamine | variably effects odorant stimulation in neurons | Rein et al. (2013) |
| X | X | *Bombyx mori* | Lepidoptera | *Bombyx mori*-cloned receptors derived from nerve tissue expressed in cultured HEK-293 cells | - | Ohtani et al. (2006) |
| X | not assessed | *Bombyx mori* | Lepidoptera | *Bombyx mori*-cloned receptors expressed in cultured HEK-293 cells | - | Ohta et al. (2004) |
| X | not assessed | *Spodoptera frugiperda* | Lepidoptera | expression in Sf9 (pupal ovarian) cell line cultures | - | Orr et al. (1992) |
| X | not assessed | *Manduca sexta* | Lepidoptera | injection of octopamine into heart tissue | increased heart rate | Prier et al. (1994) |
| not assessed | X | *Malacosoma disstria* | Lepidoptera | extracted hemolymph cell culture | - | Jahagirdar et al. (1987) |
| X | not assessed | *Locusta migratoria* | Orthoptera | isolated fat body and air sac cells | air sac cells displayed 40 fold increase in cAMP levels while fat body showed no response | Zeng et al. (1996) |
| X | not assessed | *Locusta migratoria* | Orthoptera | hemolymph and fat body homogenates | - | Orchard et al. (1982);<br>Orchard et al. (1993);<br>Wang et al. (1990) |
| X | not assessed | *Acheta domesticus* | Orthoptera | hemolymph from prothoracic legs (cAMP response inferred from phosphorylase activity) | hyperglycaemia and hyperlypaemia | Fields and Woodring (1991) |
| X | not assessed | *Aplysia spp.* | Anaspidea (Mollusca: Gastropoda) | sensory neuron-derived *Aplysia* octopamine receptors cloned and expressed in HEK-293 cells | membraine depolarization, increase in membrane excitability, action potential broadening, increased neurotransmitter release | Chang et al. (2000) |

| | | | | | | |
|---|---|---|---|---|---|---|
| X | X | *Lymnaea stagnalis* | Lymnaeoidea (Mollusca: Gastropoda) | expression of OCT receptor in HEK-293 cell culture (calcium secondary messenger activity inferred from $IP_3$ presence) | - | Gerhardt et al. (1997) |

Abbreviations: cAMP (cyclic adenosine monophosphate); PKA (protein kinase A); PKC (protein kinase C); OCT (octopamine); HEK (human embryonic kidney); $IP_3$ (inositol-1,4,5-triphosphate); Sf9 (*Spodoptera frugiperda* cell line 9); AmOA1 (*Apis mellifera* octopamine receptor 1); CHO (Chinese hamster ovary

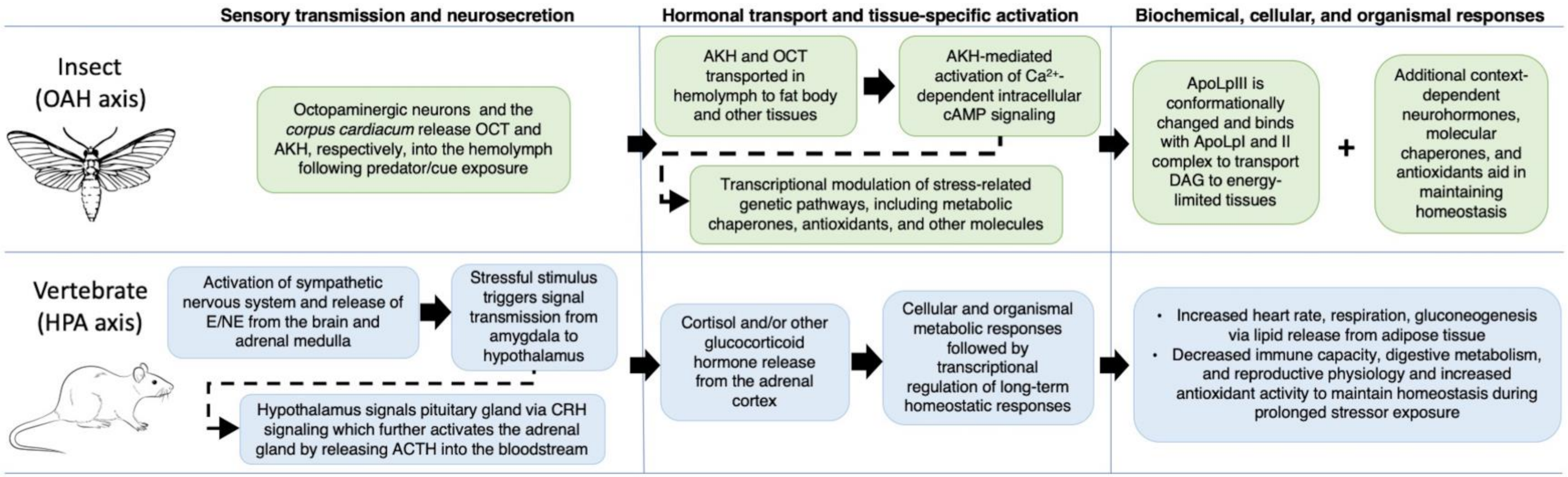


Figure 1. Biochemical and cellular stress responses in insects (top), here denoted as ‘octopamine-adipokinetic hormone axis,’ and the analogous hypothalamo-pituitary-adrenal axis (bottom) in vertebrates. Abbreviations: ACTH = adrenocorticotropic hormone, AKH = adipokinetic hormones, ApoLp = apolipophorin, $Ca^{2+}$ = calcium ion, cAMP = cyclic adenosine monophosphate, CRH = corticotropin-releasing hormone, DAG = diacylglycerol, E = epinephrine, HPA = hypothalmo-pituitary-adrenal axis, NE = norepinephrine, OAH = octopamine-adipokinetic hormone axis, OCT = octopamine.

*2-column fitting artwork

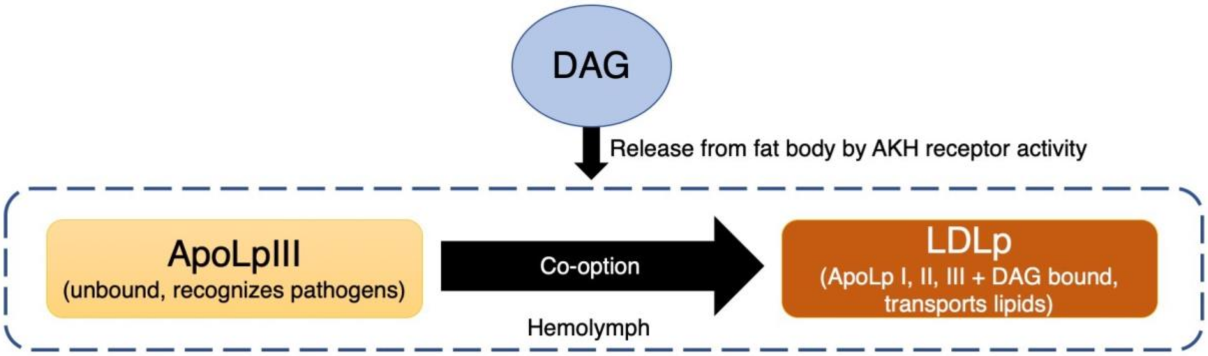


Figure 2. Conceptual diagram showing the co-option of apolipophorin III (ApoLpIII) away from immunorecognition activity towards lipid transport as a component of the low-density lipoprotein (LDLp) complex upon release of diacylglycerol (DAG) from the fat body into the hemolymph. Adapted from Adamo (2017a).

*1-column fitting artwork

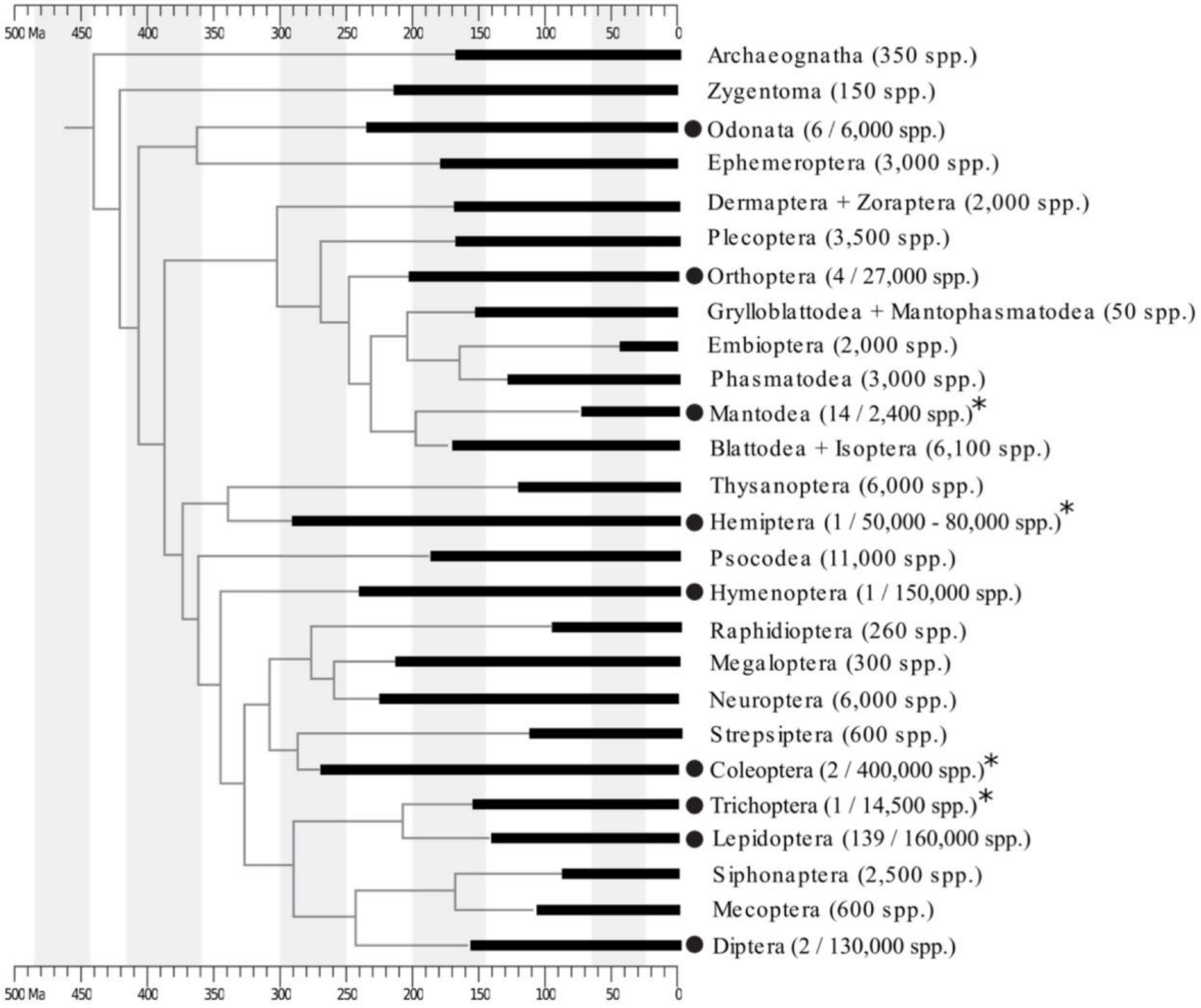


Figure 3. Evolution of insects and their taxonomic diversity in the context of studies on predator-induced stress. Tree based on Misof et al. (2014). Thick lines indicate the length of time over which that order is thought to have proliferated; circles at branch tips indicate orders in which studies of predation-related stress have been conducted, with * indicating orders that have been studied for whole-organism responses only. Numbers in parentheses indicate the number of species studied for predator-induced stress relative to known or estimated ordinal diversity.

*2-column fitting artwork

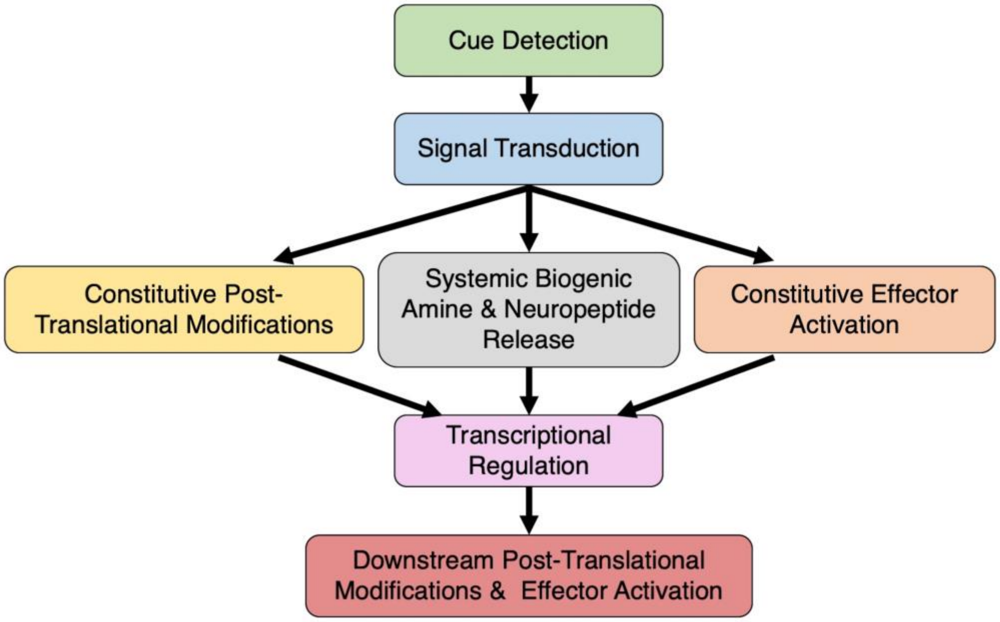


Figure 4. Flow chart describing the sequence of physiological events that occur when a prey insect perceives the presence of a predator cue.

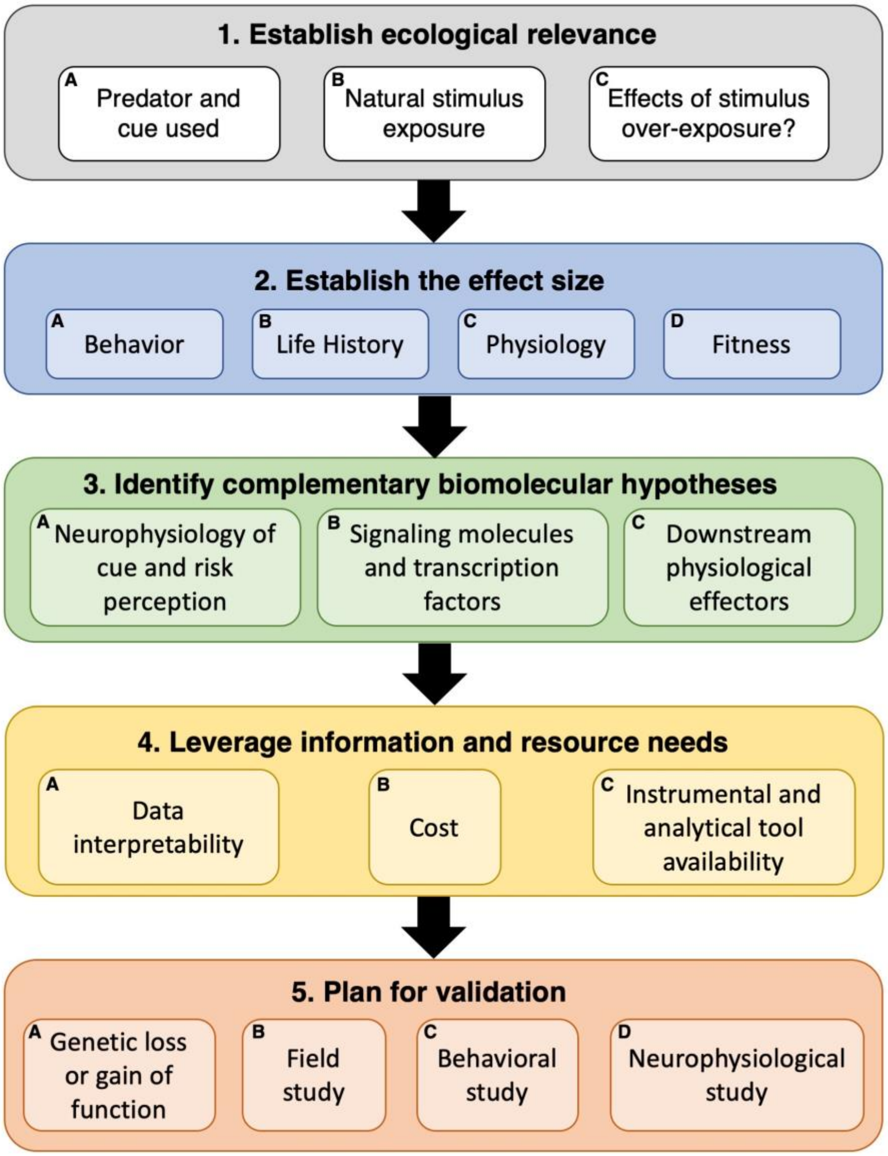
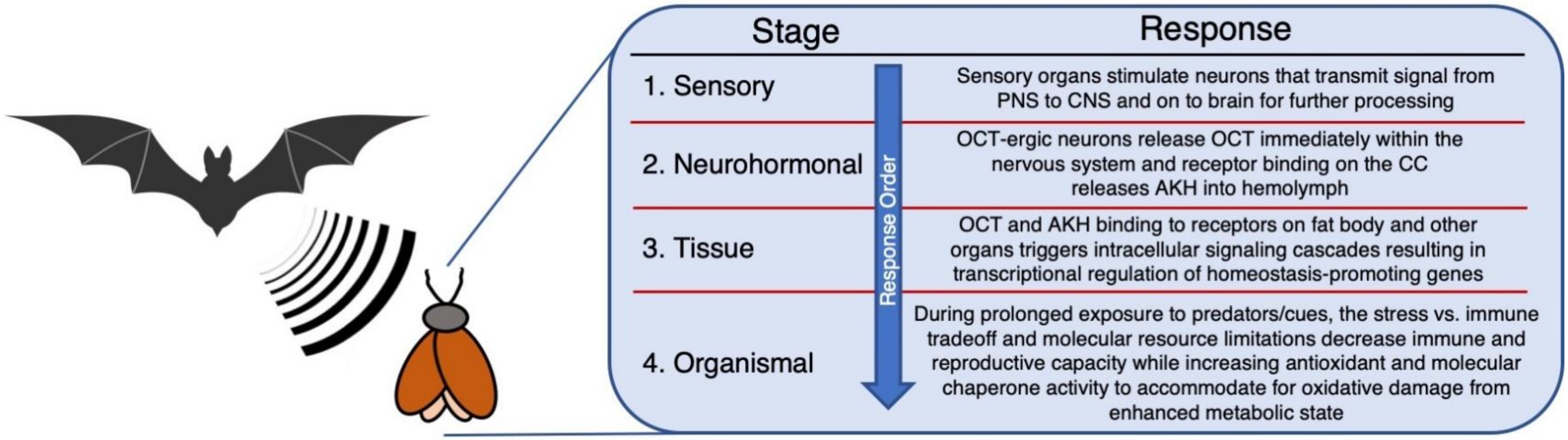


Figure 5. Protocol for studying predator-induced stress in insects, including: 1) establishing the ecological relevance of A) the predator and cue used, B) comparing experimental exposures with natural stimuli frequency, and C) accounting for the possible effects of stimulus over- or under-exposure relative to the natural stimulus frequency; 2) establishing the effect size of exposure in terms of A) behavior, B) life history, C) physiology, and/or D) fitness; 3) identifying complementary mechanistic hypotheses regarding A) the neurophysiology of cue and risk perception, B) hypothesized signaling molecules and transcription factors involved, and C) downstream physiological effector molecules; 4) leveraging information and resource needs, such as A) data interpretability, B) cost, and C) instrumental and analytical tool availability; and 5) planning for further experimental validation via A) genetic loss or gain of function experiments, B) field studies, C) behavioral studies, and/or D) neurophysiological studies.

*2-column fitting artwork



| Stage | Response |
|---|---|
| 1. Sensory | Sensory organs stimulate neurons that transmit signal from PNS to CNS and on to brain for further processing |
| 2. Neurohormonal | OCT-ergic neurons release OCT immediately within the nervous system and receptor binding on the CC releases AKH into hemolymph |
| 3. Tissue | OCT and AKH binding to receptors on fat body and other organs triggers intracellular signaling cascades resulting in transcriptional regulation of homeostasis-promoting genes |
| 4. Organismal | During prolonged exposure to predators/cues, the stress vs. immune tradeoff and molecular resource limitations decrease immune and reproductive capacity while increasing antioxidant and molecular chaperone activity to accommodate for oxidative damage from enhanced metabolic state |

Figure 6. Hypothesized biochemical and cellular stress responses to predation risk/cue perception in a generalized insect. Abbreviations: AKH = adipokinetic hormone, CC = corpora cardiaca, CNS = central nervous system, OCT = octopamine, PNS = peripheral nervous system.

*2-column fitting artwork