# Accepted Manuscript

Entomo-venomics: The evolution, biology and biochemistry of insect venoms

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Please cite this article as: Walker, A.A., Robinson, S.D., Yeates, D.K., Jin, J., Baumann, K., Dobson, J., Fry, B.G., King, G.F., Entomo-venomics: The evolution, biology and biochemistry of insect venoms, *Toxicon* (2018), doi: https://doi.org/10.1016/j.toxicon.2018.09.004.

1  Prepared as a Review for *Toxicon*
2  special issue on Arthropod Venoms
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31  Keywords: insect; venom; toxin; venom gland; venom peptide; Reduviidae; Asilidae;
32  Hymenoptera; Diptera; Neuroptera;
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**Abstract**

The insects are a hyperdiverse class containing more species than all other animal groups combined—many of which employ venom to capture prey, deter predators and micro-organisms, or facilitate parasitism or extra-oral digestion. However, with the exception of those made by Hymenoptera (wasps, ants and bees), little is known about insect venoms. Here, we review the current literature on insects that use venom for prey capture and predator deterrence, finding evidence for fourteen independent origins of venom usage among insects, mostly among the hyperdiverse holometabolan orders. Many lineages, including the True Bugs (Heteroptera), robber flies (Asilidae), and larvae of many Neuroptera, Coleoptera and Diptera, use mouthpart-associated venoms to paralyse and pre-digest prey during hunting. In contrast, some Hymenoptera and larval Lepidoptera, and one species of beetle, use non-mouthpart structures to inject venom in order to cause pain to deter potential predators. Several recently published insect venom proteomes indicate molecular convergence between insects and other venomous animal groups, with all insect venoms studied so far being potently bioactive cocktails containing both peptides and larger proteins, including novel peptide and protein families. This review summarises the current state of the field of entomo-venomics.

## 1. Multiple independent origins of venom use among insects

The >5 million species of insects estimated to exist on earth today make up the majority of eukaryotic species (May, 1988; Stork, 2018). Moreover, insect diversity goes further than just vast numbers of species. Hexapods (including insects) diverged from their closest relatives, the cave-dwelling remipede crustaceans, ~479 mya, and true insects (Ectognatha) emerged ~440 mya when they diverged from the entognathous hexapods (Collembola, Diplura and Protura) (Misof et al., 2014). Since their early evolution as one of the first animal groups to adapt to terrestrial lifestyles, the insects have undergone a spectacular evolutionary radiation and today occupy a diverse array of ecological niches.

Major adaptations powering this radiation include the early adoption of powered flight and copulation for sperm transfer by the early Pterygota, a group that includes all orders except Archaeognatha (jumping bristletails) and Zygentoma (silverfish). Holometabolous development—in which larvae must pass through metamorphosis to become adults which differ markedly in their morphology—probably further drove diversification by allowing a single species to occupy multiple niches at different life stages. Of the 34 extant orders, 18 are descended from a holometabolous ancestor that lived ~345 mya (Misof et al., 2014), including the hyperdiverse insectan orders Hymenoptera, Diptera, Coleoptera and Lepidoptera. Only one of the hyperdiverse orders, Hemiptera, is hemimetabolous. Alongside these major trends, a multitude of trophic strategies, mating systems and life histories evolved, entailing adaptations spanning the biochemical, morphological and behavioural domains.

Given this diversity, it is no surprise that adaptations such as venom and silk use have evolved independently multiple times among insects (Beard, 1963; Schmidt, 1982; Zlotkin, 1984; Sutherland et al., 2010). Envenomation, in which one animal injects another with a liquid secretion that alters its normal physiology and behaviour, is practiced by such diverse animal groups as cnidarians, molluscs, polychaete worms, nematode worms, crustaceans, arachnids, centipedes, amphibians, reptiles, and mammals (Fry et al., 2009). However, in contrast to most of these taxa, few venomous insect groups apart from Hymenoptera (ants, bees and wasps) have been studied in detail. In part, this is due to their small size, which makes it difficult to obtain large amounts of pure venom, and in part to insect hyperdiversity itself. In this review, we summarise the current state of knowledge about venoms produced by insects, focussing on the evolutionary origins of venom use, the biology surrounding venom use among major groups, and what is known of the toxins that underlie the actions of insect venoms. We report evidence for at least 14 independent origins of venom use among the insects (Fig. 1, Table 1). This number is probably greater than for all non-insect arthropods, in which venom use has evolved at least six times—in spiders, scorpions, pseudoscorpions, ticks and mites, centipedes, and remipede crustaceans (though see von Reumont et al., 2014, for discussion of possible additional venomous crustacean groups). This finding is in line with the high overall diversity of insects, and the observation that 13 out of the 14 lineages of venomous insects occur in the five hyperdiverse insect orders (Fig. 1). While almost nothing is known about most insect venoms, new discoveries about hemipteran, dipteran, hymenopteran and lepidopteran venoms are emerging, facilitated by technical advances in proteomics and next-generation nucleic acid sequencing. This study reviews what is known about the evolution, biology and biochemistry of insect venoms, and highlights opportunities for the discovery of novel toxins in hyperdiverse class.

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## 2. Diverse uses of insect venom

Most commonly (10 instances), insect venoms are used to subdue prey, with or without the additional function of deterring predators. Venoms of this type include those of predatory heteropterans in Hemiptera, many hymenopterans, the larval forms of some neuropteran and coleopteran groups, and some larval and adult forms of Diptera. Species that use venom exclusively for predator deterrence are rarer, being found only in five lineages, including some larval Lepidoptera, one group of Hemiptera (soldier aphids), a single reported species of beetle (*Onychocerus albitarsis*) as well as some hymenopteran groups in which the use of venom for predation or parasitism has been lost, including the bees (Anthophila), some ants (Formicidae) and some vespid wasps (Piek, 1986).

Injected secretions that facilitate parasitism through feeding on blood or haemolymph may also be considered venoms (Fry et al., 2009; Cabezas-Cruz and Valdés, 2014). Venoms of this type are produced by various Hemiptera, Psocodea, Siphonaptera, and multiple lineages of Diptera. Inclusion of venoms of this type would take the total number of origins of venom use in insects to at least 23, but in this review we focus on venoms used for prey capture and predator deterrence.

## 3. Venom glands and injection apparatus

Venom systems must be able to produce toxins, store them, and inject them into another animal at the appropriate time. Insects have adapted a wide range of anatomical structures to achieve these ends. Mouthpart structures and associated glands are particularly prominent, occurring in 10 out of the 14 venomous groups (Fig. 1). Only one lineage, Hymenoptera, has species that use a non-mouthpart structure for prey capture. In this case, the venom apparatus is uniquely derived from the ovipositor and sex accessory glands of the adult female (Piek, 1986). In contrast, several lineages use non-mouthpart structures to deliver venoms that are used solely for predator deterrence, including the cerambycid beetle *Onichocerus albitarsis*, which utilises modified antennae (Berkov et al., 2008), and venomous caterpillars, which utilise cuticular spines (Kawamoto and Kumada, 1984). Of the devoted defensive venoms, only that of soldier aphids is delivered using a mouthpart structure.

For mouthpart-associated systems, various arrangements of glands and injection machinery can be found. In some groups, such as Heteroptera, the venom glands are likely to be derived from, and homologous to, the salivary glands of related non-venomous species (Baptist, 1941; Walker et al., 2016). In other cases, such as the predatory larvae of horse-flies (Tabanidae), a mouthpart-associated venom gland is present that is entirely separate from the salivary glands or gut (Teskey, 1969). For most groups, it is unknown how the venom glands are related to other insect glands; for some, the gland responsible for producing the toxic secretion is uncertain. The mouthpart-associated venom glands of two groups, the assassin bugs (Hemiptera: Reduviidae) and the robber flies (Diptera: Asilidae) have recently been described in detail based on 3D reconstructions of gland structures from magnetic resonance imaging (MRI) spectroscopy and other techniques (Drukewitz et al., 2018; Walker et al., 2018b). Both groups display complex gland systems with multiple compartments, as well as muscular valves to control the flow of venom.

The heteropteran venom gland usually consists of three compartments, the anterior main gland (AMG), posterior main gland (PMG), and the accessory gland (AG). In the harpactorine assassin bug *Pristhesancus plagipennis* (Fig. 2A), the AMG and PMG were found to be active secretory tissues whereas the AG has been suggested to play a role in recycling water from the gut to the venom glands (Walker et al., 2018b). The AMG and PMG secrete and store two complex venoms, each with a distinct set of proteins and peptides. Venom produced by the PMG caused rapid paralysis and death when injected into prey insects, and was rich in proteases, suggesting it may serve the dual roles of prey capture and extra-oral digestion. We have proposed that venom produced in the AMG serves a defensive function, since it could be collected by provocation of bugs in the absence of electrostimulation. However, the function of AMG venom has not been conclusively determined. The AMG and PMG are connected to each other and to venom ducts leading to the mouthparts (vd, Fig. 2A) and AG by a two-chambered structure called the hilus. Muscular sphincter valves occur where the AMG and PMG meet the outer chamber of the hilus, and nerve fibres form neuromuscular junctions onto the muscle fibres forming the sphincter valves and surrounding the basal lamina of the main glands (Walker et al., 2018b). These structures are thought to allow the controlled release of venom from either the AMG or PMG depending on external stimuli. Additional control of injection is probably achieved using the muscle-driven venom pump within the head (vp, Fig. 2, A). For injection of venom, heteropterans use a proboscis consisting of extensively elongated maxillary and mandibular stylets (Fig. 3A–C). The outer maxillary stylets have a cutting and possibly anchoring function, whereas the inner mandibular stylets are asymmetric and interlock to form a double-barrelled needle with separate, devoted channels for venom injection and food uptake (Walker et al., 2016).

Despite showing some very different morphological adaptations, the venom system of the asilid fly *Eutolmus rufibarbis* shows some functionally convergent features with that of assassin bugs (Fig. 2B). Paralysing venom is produced in glands within the anterior dorsal thorax, called 'thoracic glands' after Kahan (1964) who found that homogenates of these glands caused paralysis and death when injected into insects. Another paired set of glands ('labial glands') exist within the labium (Whitfield, 1925) but their functional role, and whether or not they have a role in envenomation, is not yet clear. These names do not infer homology to any other insect glands, and such relationships are unclear. In any case, the thoracic glands narrow to form ducts, which fuse close to where they enter the head capsule before entering a salivary/venom pump (Drukewitz et al., 2018). This salivary pump, incorporating several sets of muscles and a one-way ring valve, has an outflow into a thin channel in the hypopharynx. It opens near the end of the hypopharynx (Fig. 2I), which is an elongated, robust and sharp structure (Fig 2H) suited to the injection of venom (Whitfield, 1925). Both robber flies and assassin bugs have mouthparts convergently adapted to form a stabbing structure with separate channels devoted to venom injection and food uptake. However, this similar functional arrangement is achieved using different anatomical substrates in each group: in Asilidae by situating the outflow of the venom apparatus on the tip of an enlarged, elongated hypopharynx, while uptaking food by a tube formed by hypopharynx and concave labium. In Reduviidae and other Heteroptera, the two tubes form as separate compartments enclosed by asymmetric, interlocking maxillary stylets.

The hymenopteran venom sting or aculeus provides an example of a non-oral venom injection structure (Fig. 2C; Fig. 3F,G). The aculeate venom apparatus and associated

structures comprises the sting, venom reservoir, and tubular venom glands that form at the base of the ninth gastral segment, plus the Dufour's gland (D'rozario, 1942). Chitinous valvilli are found on each of the lower valves of the ovipositor and sting that probably assist in the control of venom injection (Quicke et al., 1992). The venom gland consists of secretory tubular glands opening into the venom reservoir (Roat et al., 2006). Unlike the venom gland secretory cells of reduviids or asilids that are composed of columnar cells, hymenopteran venom gland secretory cells are type 3 epidermal glands (Noirot and Quennedey, 1974) with an end apparatus, owing to their derivation from sex-accessory glands which are themselves internalised epidermal structures. In Ichneumonoidea (Alves et al., 2015) and some aculeates such as Pompilidae and Vespidae (Robertson, 1968; Ratcliffe and King, 1969) a thick muscular wall surrounds the reservoir and its contraction induces venom injection (Piek, 1986). In Apidae, Formicidae and Sphecidae, only a fine reticulum of muscle fibres surround the reservoir (Robertson, 1968; Bridges Anne and Owen Michael, 1984) and venom injection has been proposed to rely on the use of a 'valve pump' mechanism (Snodgrass, 1925). The aculeus itself is slender and smooth in many species, particularly solitary and social species that are able to sting repeatedly (Baumann et al., 2018). Some social species including honeybees, some paper wasps (*Polybia* sp.) and some ants practise sting autotomy, in which the aculeus remains lodged in the victim's flesh, resulting in death for the injecting insect (Hermann, 1971). Sting autotomy is hypothesised to have evolved due to selection at the colony rather than individual level, and is thus associated with sociality. In species that practise sting autotomy, the aculeus has anchoring serrations or barbs. Such structures have developed multiple times in Hymenoptera, and display considerable morphological and functional diversity (Mulfinger et al., 1992; Zhao et al., 2015; Baumann et al., 2018).

A very different non-oral venom apparatus can be found in some caterpillars (Kawamoto and Kumada, 1984). Venom-filled spines on the exoskeleton may be derived from sensilla, or evaginations of the tegument itself. Venom-producing glands are not well-characterised, but some are likely to exist of a single large cell that secretes venom into a reservoir formed by the interior of the spine (Spadacci-Morena et al., 2016). The mechanism of venom injection is unknown, but may rely on mechanical breakage of the spine tips and either passive or active venom expulsion.

## 4. Venom biology and biochemistry

### 4.1. Hemiptera
The major radiation of venomous animals in Hemiptera is the suborder Heteroptera (venomous lineage 1, Fig. 1), which contains numerous groups that use venom to subdue prey and deter predators, such as the assassin bugs (Reduviidae), giant fish-killing water bugs (Belostomatidae), minute pirate bugs (Anthocoridae), predatory stink bugs (Asopinae) and others (Walker et al., 2016). Predatory heteropterans have complex venoms comprising enzymes, peptides, and proteins with unknown function (Walker et al., 2017; Walker et al., 2018a; Walker et al., 2018b). Many families of venom proteins are found in both Reduviidae and Belostomatidae, which span much or all of the phylogenetic diversity in Heteroptera, leading us to propose that many of these were recruited into venom anciently in heteropteran evolution (Walker et al., 2018a). Heteropteran venoms are known to cause paralysis, tissue liquefaction and death when injected into invertebrates (Edwards, 1961; Zerachia et al., 1973a; Maran and Ambrose, 2000; Maran et al., 2011; Walker et al., 2018b), and pain and sometimes neurotoxic

249 symptoms and/or death when injected into vertebrates (Zerachia et al., 1973a; Haddad
250 et al., 2010). Assassin bug venom is also known to have a strong cytolytic effect on both
251 insect and mammal cells (Edwards, 1961; Zerachia et al., 1973b) which may have
252 masked detection of its other pharmacological effects. We found that venom produced in
253 the PMG of the assassin bug *Pristhesancus plagipennis* rapidly paralyses crickets within
254 several seconds (Walker et al., 2018b). This finding, together with the high potency
255 (Edwards, 1961) and reversible paralytic effects (Zerachia et al., 1973a) of assassin bug
256 venom under some circumstances suggest the presence of specific neurotoxins.
257 However, the only heteropteran toxin with a known neurotoxic activity is Ptu1 from the
258 assassin bug *Peirates turpis*, an inhibitor cystine knot (ICK) peptide (Bernard et al.,
259 2001) that inhibits $Ca_V2.2$ voltage-gated calcium channels (Corzo et al., 2001). Possibly,
260 membrane-disrupting toxins contribute to prey paralysis, as has been observed in
261 Hymenoptera (Robinson et al., 2018). The redulysins, a family of abundant toxins in *P.
262 plagipennis* venom, probably form pores similar to trialysin, a member of the same
263 family that has been isolated as a minor component from venom of the kissing bug
264 *Triatoma infestans*. Trialysin is activated through N-terminal cleavage by a serine
265 protease (Martins et al., 2008) and forms a voltage-dependent pore in lipid bilayers
266 (Amino et al., 2002). The haemolysin-like proteins, as their name indicates, have been
267 ascribed a putative cytolytic function based on similarity to bacterial haemolysins
268 (Assumpção et al., 2008), though we note that the sequence similarity between these
269 groups is quite low (no hits with E < 0.1 when heteropteran hemolysin-like proteins are
270 searched against GenBank's nr database).

272 Other components in heteropteran venoms may be involved in liquefaction of prey
273 tissues. Proteases in the C1A, A1A, and M12 families, and especially the abundant S1
274 family, as well as enzymes such as hyaluronidase, chitinase and nuclease, are likely to
275 degrade biopolymers and assisting both the spread of other toxins and liquefaction. In
276 addition, the identification of venom proteins in both reduviids and belostomatids
277 annotated as gelsolins and fasciclins/periostins suggests more specific mechanisms of
278 prey liquefaction: gelsolin, as the most potent actin depolymerisation protein known
279 (Sun et al., 1999) may assist by deconstructing cytoskeletons; fasciclins/periostins,
280 which have cell adhesion domains (Clout et al., 2003), may disrupt cell adhesion.
281 However, many of the most abundant proteins in heteropteran venoms cannot be
282 assigned any putative function based on their sequence. These include the CUB domain
283 proteins, and heteropteran venom proteins classified into families 1–34 (Walker et al.,
284 2017; Walker et al., 2018b). Of these, the CUB domain proteins and families 1 and 2 are
285 the main non-protease components of *P. plagipennis* venom harvested by
286 electrostimulation (Walker et al., 2017).

288 A separate group of venom-using hemipterans are the soldier aphids (venomous lineage
289 2, Fig. 1), which are wingless and sterile clones produced by parthenogenesis to defend
290 the colonies of some social aphids in the families Hormaphididae and Pemphigidae
291 (Stern and Foster, 1996). Soldiers attack insects that stray near their communal gall by
292 piercing them with their proboscis. In all cases of *Ceratovaeuna nekoashi* soldiers
293 attacking caterpillars observed by Kurosu and Aoki (1988b), this led to paralysis and/or
294 death of the insect. Bites of humans produce an itching sensation. A subtractive cDNA
295 library comparing soldiers and non-soldiers of *Tuberaphis styraci* (Kutsukake et al.,
296 2004) identified a cathepsin B homologue as a major soldier-specific protein, with
297 expression levels >2,000 times higher in soldiers vs. non-soldiers. This protein could be

7

298 detected in the haemolymph of caterpillars paralysed and killed by soldiers, a process
299 that took some 30 min. Although heteropterans produce venom toxins in salivary glands
300 (Walker et al., 2018b), Kutsekake et al. found that *T. styraci* venom cathepsin B was
301 produced in the midgut, and may be regurgitated into prey.
302
303 **4.2. Hymenoptera**
304 Order Hymenoptera (sawflies, wasps, ants and bees; venomous lineage 3, Fig. 1) is a
305 diverse order with over 150,000 extant venomous species, including solitary and social
306 species with a variety of life history strategies including parasitoidism, predation, and
307 pollen-feeding (Davis et al., 2010). Venom use has been adapted to facilitate each of
308 these unique life histories, producing a wide range of venom biochemistries. Compared
309 with venoms from other insect groups, venoms of the Hymenoptera have been the
310 subject of extensive study. Here, we provide a general overview and refer the reader to
311 other works for more detailed information (Piek, 1986; Aili et al., 2014; Moreau and
312 Asgari, 2015; Konno et al., 2016; Lee et al., 2016; Touchard et al., 2016a; dos Santos-
313 Pinto et al., 2018).
314
315 Venom use arose in Hymenoptera associated with parasitic oviposition. In the most
316 basal hymenopterans, the sawflies (paraphyletic Symphyta), the ovipositor is used to cut
317 into plants where the eggs are laid. To facilitate parasitism, *Sirex noctilio* (Siricidae)
318 injects both a symbiotic white-rot fungus (*Amylostereum areolatum*) and venom
319 containing an 11-residue glycopeptide called noctilisin into host plants (Bordeaux et al.,
320 2014).
321
322 Suborder Apocrita transitioned from parasitic oviposition of plants to parasitic
323 oviposition of invertebrates. Eggs of parasitoid species are laid either in or on an
324 envenomated host. The most common effects of venom are immunosuppression,
325 developmental arrest, paralysis and metabolic changes (Asgari, 2012). For example,
326 venom of the ectoparasitoid *Nasonia vitripennis* induces neuronal cell death,
327 immunosuppression, inhibition of haemocyte aggregation, and developmental arrest
328 (Rivers et al., 1993), partly mediated by transcriptional modulation of the enhancer-of-
329 split complex signalling pathway (Martinson et al., 2014). Venom of the endoparasitoid
330 *Pimpla hypochondriaca* (Apocrita: Ichneumonidae) includes a 33 kDa haemocyte
331 aggregation inhibitor that suppresses host encapsulation responses (Richards and Dani,
332 2008) and a 22 kDa heterodimeric paralysis-inducing protein, pimplin (Parkinson et al.,
333 2002). These venoms are complex cocktails of many proteins and peptides, including
334 many of high molecular mass (Parkinson et al., 2003; Parkinson et al., 2004; Danneels et
335 al., 2010; Moreau and Asgari, 2015). Uniquely, symbiotic viruses (polydnaviruses, PDVs)
336 are important functional components of venoms of the Braconidae and Ichneumonidae,
337 which independently integrated two different viruses into their genomes (Dupuy et al.,
338 2006; Herniou et al., 2013). These integrated proviruses produce encapsulated virions
339 present in venom that facilitate or are essential for successful parasitism (Asgari, 2012).
340
341 In one apocritan group, Aculeata (Latin "the stingers"), the ovipositor lost its egg-laying
342 function. With eggs exiting at the base of the structure, the remainder became a devoted
343 venom injection apparatus. This innovation, coupled with multiple origins of sociality
344 and trophic switches, fuelled diversification into yet more ecological niches.
345 Nevertheless, most aculeate species are solitary parasitoid wasps that use venom to
346 partly or completely immobilise prey fed to larvae within a sealed nest or burrow. In

347 these species, defensive envenomation usually plays a secondary role (Piek, 1986). In
348 contrast, defence is the primary role of venom for many social aculeates, especially those
349 that provision larvae with other food sources, such as the bees (Anthophila), some ants
350 (Formicidae), and some vespid wasps.

352 In both solitary and social Aculeata, the major toxin class is amphipathic, cationic, α-
353 helical peptides. A prime example is melittin (Fig. 4, A), a 26-residue peptide that is the
354 major component (comprising ~50% of the dry weight) of venom from the honeybee
355 *Apis mellifera*. Melittin causes the immediate, intense pain of honeybee stings by
356 disrupting lipid bilayers, including those of pain-sensing neurons (nociceptors). Similar
357 peptides with membrane-disrupting activities are widespread in Aculeata, including the
358 bombilitins, mastoparans, vespid chemotactic proteins, and others (Piek, 1986). Some of
359 these amphipathic α-helical peptides have been reported to have more specific
360 pharmacological effects, though we note that some may reflect indirect effects of
361 membrane disruption. Wasp kinins have very similar structures to vertebrate kinin, a
362 nonapeptide hormone involved in pain signalling, and may induce pain in mammals by
363 interacting with bradykinin receptors (Pisano, 1979). Inhibition of cholinergic
364 transmission in insects is also reported for kinin-like peptides from venom of the flower
365 wasp (Scoliidae) *Colpa interrupta* (Piek et al., 1990). Delayed inactivation of voltage-
366 gated sodium channels, which may induce pain by causing persistent firing of
367 nociceptors, is caused by α-and β-pompilidotoxins from venoms of the spider wasps
368 (Pompiliidae) *Anoplius samariensis* and *Batozonellus maculifrons* (Konno et al., 1998;
369 Sahara et al., 2000), as well as poneratoxin isolated from the highly painful venom of the
370 bullet ant *Paraponera clavata* (Piek et al., 1991; Johnson et al., 2017).

372 Some aculeate venom peptides combine as homomeric or heteromeric dimers. For
373 example, ectatomin-1 (Fig. 4B), from the venom of the ant *Ectatomma tuberculatum*, is a
374 heteromeric dimer linked by an interchain disulfide bond, with an additional internal
375 disulfide bond in each chain (Nolde et al., 1995; Pluzhnikov et al., 1999). Mp1a, from
376 venom of the jack jumper ant *Myrmecia pilosula,* has another arrangement, with the two
377 antiparallel chains linked by two interchain disulfide bridges (Dekan et al., 2017). Both
378 ectatomin-1 and Mp1a disrupt lipid bilayers, though Ectatomin-1 has also been reported
379 to inhibit $Ca_V1$-type calcium channels at nanomolar concentrations (Arseniev et al.,
380 1994). Despite the presence of disulfide bonds, these dimers retain the amphipathic α-
381 helical structure of the aforementioned linear peptides. The shared structure of the
382 genes encoding all of these amphipathic α-helical peptides suggests that most, or all, are
383 products of a single large superfamily, dubbed the aculeatoxins (Robinson et al., 2018).

385 Other disulfide-rich peptides in hymenopterean venom probably have separate origins
386 to the aculeatoxin superfamily. Apamin, mast cell degranulating peptide, and tertiapin
387 (Fig. 4C) are members of a two-disulfide-containing peptide family which are minor
388 components of the venoms of bees, of which all known members inhibit some type of
389 potassium channel (Lazdunski, 1983; Kondo et al., 1992; Jin and Lu, 1998). The
390 poneritoxins, abundant components of the venom of *Anochetus emarginatus* which
391 includes the $Ca_V1$ inhibitor Ae1a (Fig. 4, D; Touchard et al., 2016b), are another two-
392 disulfide-containing peptide family with a distinct structure. Three-disulfide-containing
393 peptides, common in venoms of other animals (Norton and Pallaghy, 1998), are rare in
394 Hymenoptera. An epidermal growth factor (EGF)-like peptide of unknown function with
395 three disulfide bonds is a major component of giant red bull ant (*M. gulosa*) venom

(Robinson et al., 2018), and a dendrotoxin-like peptide has been reported as a minor component of venom of the potter wasp *Eumenes pomiformis* (Baek and Lee, 2010). ICKs have been suggested to exist in some aculeate venoms (Torres et al., 2014; Kazuma et al., 2017), but proteomic evidence is lacking. Larger proteins are frequently present in aculeate venoms, especially phospholipase $A_2$, hyaluronidase, lipase, CRiSP (cysteine-rich secretory protein), and acid phosphatase (Schmidt et al., 1986).

Non-proteinaceous toxins are also important functional components of some aculeate venoms. The philanthotoxins, polyamines found in venom of the beewolf *Philanthus triangulum* (Crabronidae), cause paralysis by blocking excitatory ligand-gated ion channels at neuromuscular junctions (Piek, 1982; Eldefrawi et al., 1988). Another example comes from the jewel wasp, *Ampulex compressa*, which injects venom into the ganglia and brain of cockroaches. Jewel wasp venom contains high levels (10–30 mM) of the inhibitory transmitter γ-aminobutyric acid (GABA), as well as the GABA receptor agonists taurine and β-alanine (Weisel-Eichler et al., 1999; Moore et al., 2006). The resulting activation of inhibitory GABAergic transmission contributes to the hypokinetic state induced by envenomation that allows the wasp grub to consume the living cockroach. Some ants also have venoms that are primarily non-proteinaceous. The venom of fire ants (*Solenopsis* sp., Myrmecinae) consists mostly of hydrophobic piperidines called solenopsins (MacConnell et al., 1971) that have insecticidal, cytolytic, and antibacterial activities (Blum et al., 1958), while ants of the subfamily Formicinae have venoms composed primarily of formic acid, which they spray through an acidopore for defence.

### 4.3. Neuroptera

The larvae of most Neuroptera (lacewings, antlions, owlflies and allies; venomous lineage 4, Fig. 1) are venomous predators of other insects (Tauber et al., 2009). Their hunting strategies range from active searching to ambush, notably including the pitfall traps employed by many antlions (Myrmeleontidae), which inspired the fictional sarlacci of the *Star Wars* universe. Prey impaled by the pincer-like mouthparts usually cease movement within seconds, and remain paralysed even when separated from the neuropteran larva immediately after prey capture (Henry, 1977; Canard, 2001). Venom is also postulated to have a liquefying function, since (as with reduviids, asilids and others) food can only be ingested in liquid form. Venom is also used defensively and causes pain in envenomated humans (Schmidt, 1982; Ronald Jenner, personal communication).

The source of the venom responsible for prey paralysis by neuropteran larvae is unclear from current literature. On one hand, numerous morphologists have described in detail a gland within the maxilla that is termed the venom gland (Wundt, 1962; Rousset, 1966; Gaumont, 1976; Canard, 2001; Beutel et al., 2010a; Beutel et al., 2010b; Randolf et al., 2014). This gland (Fig. 5) exists within the medio-dorsal portion of the base of the maxilla and consists of column-like cells with large nuclei resembling secretory cells (Gaumont, 1976). The lumen of the gland thins as it progresses anteriorly through a 'venom channel', emerging at a pore close to the tip of the maxilla. It is present in diverse predatory neuropteran families, including the basal Nevrorthidae, but not the related non-venomous orders Megaloptera and Raphidioptera. The maxillary gland has been secondarily lost in the Sysiridae, which are not predators of arthropods but instead feed on aquatic sponges and bryozoans (Gaumont, 1976; Beutel et al., 2010a). The anatomical

arrangement of the gland and its delivery canal, as well as its presence in venomous but not non-venomous taxa led these researchers to conclude that it performs a role in prey capture.

There are also suggestions that the alimentary canal might be the source of paralysing venom produced by larvae. Larval neuropterans take up food through a food canal (fc, Fig. 5) formed by interlocking mandibles and maxillae. Unlike in assassin bugs or robber flies, this food canal is a paired structure, as each 'pincer' is formed by the maxilla and mandible on either side of the body (Fig. 3M). The food canal emerges at the tip of the pincer (i.e. close to where the maxillary gland empties, in the part that penetrates into prey). Evidence that the gut might be responsible for producing paralytic factors comes from a study by Henry (1977), who injected soluble extracts of different body parts of the owlfly larva *Ululodes mexicana* into cockroaches (*B. germanica*). Whereas head and prothorax extracts caused paralysis in seconds, a 'jaw extract' (probably containing the maxillary glands) produced only weak effects.

Other evidence that the alimentary canal might be involved in producing larval venom toxins comes from a series of publications focussing on toxins produced by bacterial symbionts in the gut of the antlion *Myrmelion bore* (Matsuda et al., 1995; Yoshida et al., 1999; Yoshida et al., 2001; Nishiwaki et al., 2004; Nishiwaki et al., 2007a; Nishiwaki et al., 2007b). Larval neuropterans have a 'dead-end' digestive system, disconnected from the anus that is adapted for silk production (Weisman et al., 2008), which may provide unique conditions for symbiosis. In the first of this series (Matsuda et al., 1995), venom was collected from the tips of the mandibles (i.e., probably originating either in the alimentary canal or the maxillary gland) of the antlion *Myrmeleon bore*. Venom obtained in this way was found to contain a paralytic toxin that was inactivated by heat or protease treatment, consistent with it being proteinaceous. After size exclusion and ion exchange chromatography, the purified toxin was found to be have a molecular mass of 167 kDa. It was potently paralytic, having a minimum paralysing dose of 40 ng per cockroach. This finding is in contrast to the usually small peptides and alkaloids used by most arthropod groups to paralyse prey (Fry et al., 2009), and a previous finding that neuropteran paralytic toxins are resistant to proteases (Henry, 1977). The N-terminal amino acid sequence of this 'AMLB toxin' was elucidated and used to detect its presence in the thorax and abdomen of larvae, but not other life stages or the head of larvae (Yoshida et al., 1999). Other studies focussed on bacterially-encoded toxins that were isolated from media used to culture bacterial strains isolated from the antlion's esophagus and crop (Yoshida et al., 2001; Nishiwaki et al., 2004; Nishiwaki et al., 2007b) or the bacteria themselves (Nishiwaki et al., 2007a). These include a sphingomyelinase C from *Bacillus cereus* (Nishiwaki et al., 2004), 'sphaericolysin' from *B. sphaericus* (Nishiwaki et al., 2007b), and a homologue of the *E. coli* chaperonin GroEL from *Enterobacter aerogenes* (Yoshida et al., 2001). However, while these toxins are potently insecticidal and they have been interpreted to contribute to prey paralysis by antlions in nature, the actual extent of this contribution is unknown. To clarify venom use by larval neuropterans, it would be desirable to examine the contents of both the maxillary gland and digestive tract using transcriptomics and proteomics, to isolate and characterise toxins from both tissues, and to determine which toxins are actually injected and facilitate prey capture.

**4.4. Coleoptera**

Schmidt (1982) lists diverse coleopteran lineages as having venomous larvae, including the fireflies (Lampyridae), diving beetles (Dytiscidae), whirligig beetles (Gyrinidae), ground beetles (Carabidae), tiger beetles (Cicindelidae), water beetles (Hydrophilidae), and carrion beetles (Silphidae).

Lampyridae such as *Lampyris noctiluca* and *Photuris pennsylvanica* attack prey such as snails and earthworms, by injecting a rapidly paralysing and liquefying venom (Williams, 1917; Fabre, 1924). Snails envenomated by *L. noctiluca* larvae—but removed before feeding takes place—recover from paralysis after several days, suggesting neurotoxins rather than tissue damage is responsible for paralysis (Fabre, 1924). Similar rapid paralysis has been noted after bites from staphylinoideans such as the silphid *Phosphuga atrata* and adephagans such as the dytiscidan *Lancetus marginatus* (Heymons et al., 1927; Balduf, 1935).

The Lampyridae, Dytiscidae, Gyrinidae, and Carabidae have grooves or tubes running through their mandibles that are used to inject venom into impaled prey (Balduf, 1935). Silphidae and Hydrophilidae lack such tubes, but have been reported to inject similar venoms into wounds inflicted with the mandibles (Richmond, 1920; Heymons et al., 1927; Balduf, 1935). Since (like other coleopteran larvae) none of these groups possess salivary glands, the venom responsible for paralysis and death has been suggested to be produced in the alimentary canal or an associated structure (Balduf, 1935). If so, single delivery tube is probably used for injecting venom and uptaking food. Evidence of a true venomous nature is best for Lampyridae, Dytiscidae, Gyrinidae, Carabidae and Silphidae, but more limited for Hydrophilidae.

Despite the many similarities between the above groups in the way that venom is produced and delivered, consideration of the phylogeny of Coleoptera, the most diverse insect order (Hunt et al., 2007; McKenna et al., 2015), suggests multiple origins of venom use. We tentatively group them into three lineages: Adephaga (containing Dytiscidae, Gyrinidae, Carabidae and others), venomous lineage 5, Fig. 1); the clade Staphylinoidea (containing Silphidae) + Hydrophiloidea, lineage 6; and Lampyridae (and possibly other Elateroidea), lineage 7.

In addition to these three groups with evidence of venom use in the larval stage, a single species of longhorn beetle (Cerambycidae) has been reported to use venom (Smith, 1884; Berkov et al., 2008). Unlike the groups described above, *Onychocerus albitarsis* (venomous lineage 8, Fig. 1) produces and delivers venom using the terminal segment of its antenna for defensive purposes. The terminal segment resembles a scorpion's stinger and carries a dual pore for the injection of venom (Fig. 3J–L). In humans, envenomation by *O. albitarsis* produces pain and inflammation, similar to a honeybee sting (Berkov et al., 2008).

### 4.5. Lepidoptera
Larval lepidopterans (caterpillars) use venom to deter predators. The chief symptom of envenomation is pain and/or irritation, though some caterpillar envenomations of humans are capable of causing numbness, vomiting, respiratory paralysis, and even death (Kawamoto and Kumada, 1984; Balit et al., 2003; Hossler, 2010; Maggi and Faulhaber, 2015).

543 The best-characterised lepidopteran venom is that of South American saturniids in the
544 genus *Lonomia*. Like other Hemileucinae (venomous lineage 9, Fig. 1), *Lonomia* sp.
545 possess spines that inject painful venom. However, the venom of *Lonomia* sp. is unusual
546 among both hemileucines and other caterpillars in causing disturbances to the blood
547 system that can prove fatal. Envenomation produces local effects such as burning pain
548 sensations and swelling, followed by systemic consumptive coagulopathy that produces
549 haemorrhagic syndrome. In severe cases, pulmonary and intracranial haemorrhage and
550 renal failure result in death (Alvarez-Flores et al., 2010). Proteomics and
551 transcriptomics of the venom spines suggest that venom has a complex composition
552 containing numerous peptides and proteins (Veiga et al., 2005; Ricci-Silva et al., 2008),
553 and the biological effects are likewise complex. Toxins such as the hemolin-like Losac
554 (Alvarez-Flores et al., 2011) and the lipocalin Lopap (Reis et al., 2006) are known to
555 activate factor X and prothrombin respectively, causing dispersed intravascular clots.
556 These are then degraded, leading to a consumption of coagulation factors and inability
557 of the blood to clot. Venom has additional actions on vascular muscle cells, inducing
558 proliferation and migration that may contribute to haemorrhage (Moraes et al., 2017).
559 Several recent reviews provide a comprehensive overview of *Lonomia* venom (Carrijo-
560 Carvalho and Chudzinski-Tavassi, 2007; Alvarez-Flores et al., 2010; Maggi and
561 Faulhaber, 2015).
562
563 Zygaenoidea (venomous lineage 10, Fig. 1) is another lepidopteran group containing
564 species with powerful liquid venoms. Larvae of flannel moths (Megalopygidae), known
565 as 'puss caterpillars', 'asps' or 'toxic toupees', possess stinging spines hidden by longer
566 non-venomous hairs (Deml and Epstein, 2001b). *Megalopyge opercularis* is a North
567 American species with a particularly severe sting that results in intense pain radiating
568 from the site of envenomation to other parts of the body, as well as red welts. Other
569 reported symptoms are itch, numbness, chest pain, tingling sensation, headache, fever,
570 vomiting, tachycardia, shock-like syndrome, and convulsions. The pain is described as
571 'hot coals applied to the skin' or 'being hit by a baseball bat' (McMillan and Purcell, 1964;
572 Pinson and Morgan, 1991; Stipetic et al., 1999; Eagleman, 2008). Megalopygid venom
573 has not been characterised at the molecular level but it is proposed to be proteinaceous
574 based on the observation that it is rapidly inactivated by boiling, or by heating at 55°C
575 for one hour (Foot, 1922). A venom spine extract injected into mice resulted in pain-like
576 behaviours, bristling, profuse defecation, difficulty walking, and death after one hour
577 (Foot, 1922). The venom of *Megalopyge urens* has been shown to have hyaluronidase
578 and protease but not phospholipase $A_2$ or nuclease activity, and to have little effect on
579 blood coagulation (Ardao et al., 1966). Hydroquinone, nicotine, and isopropyl miristate
580 were also identified in venom of an unidentified Venezualan megalopygid (Deml and
581 Epstein, 2001a; Deml and Dettner, 2003).
582
583 Another group of zygaenoidean caterpillars, the Limacodidae, are known as slug moth or
584 cup moth caterpillars. The venomous species (about half of the ~1,650 described
585 species) comprise a single monophyletic family and are known as 'stinging nettles'
586 (Epstein, 1996; Zaspel et al., 2016). The remaining species, termed 'jellies' or 'monkey
587 slugs', are non-venomous. Venom of most Limacodidae induces immediate severe pain
588 that fades over several hours. Injection of a venom spine extract of *Parasa consocia* into
589 mice produces pain behaviours, shivering, and limb paralysis (Kawamoto, 1978b).
590 Fractionation of the extract into high (>15 kDa) and low (<15 kDa) molecular mass
591 fractions, followed by application to wounds of human volunteers, revealed that both

592 fractions cause pain. Whereas the low-mass fraction caused immediate pain that passed
593 quickly, the high-mass fraction caused slow-onset, lingering pain. Further size
594 fractionation of the low-mass fraction revealed multiple pain-causing substances
595 including histamine and a 'peptide of several thousand [Daltons]' (Kawamoto, 1978b).
596 This peptide was suggested to be kinin-like due to its ability to contract guinea pig ileum
597 without tachyphylaxis and to increase vascular permeability.

599 The phylogenetic spread of caterpillars that are venomous, and the number of
600 independent origins of venom use in Lepidoptera, is clouded by lack of information
601 about how the various kinds of cuticular structures are related, and which of these
602 constitute toxin delivery systems as opposed to mechanical defences. Kawamoto and
603 Kumada (1984) classified these structures into ten types of spicules and spines, eight of
604 which are described as venom-injecting. According to this publication, the spicules of
605 many species that produce irritating effects on skin, including many in Thaumetopoinae
606 and Erebidae (Noctuoidea) and Lasiocampoidea, possess internal structures for the
607 delivery of toxins. The case for injected toxins in these groups is supported by findings
608 such as those of Lamy et al. (1986), who found that skin irritation produced by contact
609 with the processionary caterpillar *Thaumetopoea pityocampa* is likely due to a 28 kDa
610 heterodimer, thaumetopoein, that was purified from spicule extracts. Similarly, soluble
611 extracts of spicules from the irritative brown-tail moth caterpillar *Euproctis subflava*
612 (Erebidae) contains irritative enzymes, histamine, and kinin-like substances
613 (Kawamoto, 1978a; Kawamoto et al., 1978). Further investigations are required to
614 clarify if these groups warrant being called venomous, and if so, whether they have a
615 common evolutionary origin. In this review, we have focused for simplicity on groups
616 that definitely inject potent liquid venoms, which comprise the Megalopygidae and
617 Limacodidae within Zygaenoidea, and the hemileucine Saturniidae within Bombycoidea.
618 By the most conservative estimate, venom use has evolved in Lepidoptera twice, though
619 the number may be higher if venom use has separate origins in Megalopygidae and
620 Limacodidae, and/or if additional noctuoid or lasiocampoid groups are counted
621 separately.

### 4.7. Diptera
624 Many of the True Flies are predatory, especially in larval stages (Wiegmann et al., 2011).
625 Of these, there is evidence that several groups inject bioactive venoms into prey or to
626 deter predators (Schmidt, 1982; von Reumont et al., 2014). Here, we discuss those
627 groups that use venom for predation and defence.

629 The best-characterised dipteran venom is that made by robber flies (Asilidae, venomous
630 lineage 11, Fig. 1). Adult Asilidae hunt flying prey, which is incapacitated using venom
631 before being consumed. Venom likely originates in the thoracic glands, since extracts of
632 these produce swift paralysis when injected into prey insects (Whitfield, 1925; Kahan,
633 1964; Musso et al., 1978). Analysis of thoracic gland protein extracts and transcriptomes
634 from the robber flies *Eutolmus rufibarbis* and *Machimus arthriticus* suggests their venom
635 is a complex mixture of proteins and peptides (Drukewitz et al., 2018). Of these,
636 neurotoxic activity has been demonstrated for the *M. arthriticus* peptide U-Asiliditoxin-
637 Mar-1a. Mar-1a has a primary structure suggesting it takes the ICK fold that has been
638 widely recruited in animal venoms (Norton and Pallaghy, 1998; Undheim et al., 2015).
639 When injected into the ocelli of bees, Mar-1a made by solid-phase synthesis produced
640 symptoms of disorientation and paralysis, suggesting that venom peptides contribute to

the observed neurotoxicity of asilid venom. The functions of most other proteins in the venom, many of which are of higher molecular mass, remain unknown. Few of the identified proteins are annotated as enzymes, in contrast to other species that use salivary venoms suggested to liquefy prey (Walker et al., 2017). The larvae of Asilidae, as well as many other asiloidean families, are predatory. These larvae kill prey quickly and efficiently, possibly with the assistance of salivary gland secretions injected into the prey via grooves on the surface of the mouthhooks (Sinclair B, 1992).

The Tabanomorpha (march flies, horse flies and allies; venomous lineage 12, Fig. 1) are another dipteran group with potently bioactive venoms. In contrast to Asilidae, in Tabanomorpha it is the larvae that are venomous predators, whereas the adults feed on nectar or blood. Tabanid larvae occur typically in water or mud, where they are voracious predators, including of invertebrates with potent chemical defences such as bombardier beetles and small vertebrates such as toads (Jackman et al., 1983; Nowicki and Eisner, 1983). Prey impaled by the mandibles make a few violent movements before succumbing to paralysis (Schremmer, 1951; Teskey, 1969). Larvae also bite humans when they come into contact, producing pain, irritation and itch (Otsuru and Ogawa, 1959; Jackman et al., 1983). Venom is delivered through a canal in the mandible that exits close to the tip. This canal is connected to a gland within the head (close to the anterior margin of the cibarial pump) that is entirely separate from the alimentary canal (Schremmer, 1951; Teskey, 1969; Woodley, 1989). Although Tabanomorpha and Asiloidea are closely related within a basal clade of suborder Brachycera (Wiegmann and Yeates, 2017), they use differing anatomical structures for venom injection, and are venomous in different life stages. Adult Asilidae inject venom made in thoracic glands through a duct in the hypopharynx, whereas larval (but not adult) Tabanomorpha inject venom through mandibular canals. Since these mandibular venom canals are present in Tabanidae, Athericidae and Pelecorhynchidae, but not Rhagionidae (Woodley, 1989; Sinclair B, 1992; Courtney et al., 2000), venom use by Tabanomorpha may be confined to these former families, and likely has separate origins to that in Asilidae. Alternatively, the use of salivary-gland-derived venom might be widespread among larvae of Brachycera that are predatory, including various Tabanomorpha, Asiloidea and Empidoidea, but has diverged into highly different forms in Tabanidae and Asilidae.

Among the diverse Muscomorpha, larvae of some of the snail- and slug-killing flies (Sciomyzidae; venomous lineage 13, Fig. 1) have potent venoms used for predation. Envenomation of a slug by *Tetanocera plebeia* causes quivering and paralysis after 60 s. If feeding is prohibited, the paralysis is reversible, with the time to recovery depending on the length of the bite (Trelka and Berg, 1977). Bites of 15 s duration were sufficient to paralyse slugs for one hour. The venom is proposed to originate from salivary glands, and the paralytic molecule has been reported to have been isolated and characterised as a high molecular mass protease (Berg and Knutson, 1978). However, this finding has been called into question by von Reumont et al. (2014) on the grounds that venom neurotoxins are more often small molecules or peptides rather than enzymes.

A final example of Diptera that are venomous in the larval stage is *Aphidoletes aphidimyza* (Bibionomorpha: Cecidomyiidae; venomous lineage 14, Fig. 1). Envenomation of aphid prey produces paralysis in a few minutes. The most likely source of paralysing venom is the salivary glands, injection of a homogenate of which recapitulates the observed paralysis (Mayr, 1974; von Reumont et al., 2014). The extent

of venom use in this lineage is unknown, but it might extend to other Bibionomorpha that are predatory in the larval stage.

## 5. Conclusions

Consistent with their high overall diversity, we find evidence for 14 groups of insects that have independently evolved venom use for prey capture and predator deterrence. All except one of these (Neuroptera) occurs in the hyperdiverse orders Hemiptera, Hymenoptera, Coleoptera, Lepidoptera, and Diptera. Most groups use mouthpart-associated structures to subdue and liquefy prey, while defensive venoms are often associated with non-mouthpart structures.

Hymenoptera is the only lineage of venomous insects in which detailed structural and functional characterisation of toxins has been applied to more than a few species. Most hymenopteran venom toxins are membrane-disrupting α-helical peptides without disulfide bonds, but a range of dimeric and/or disulfide-bonded peptides, as well as enzymes and small molecules, are used to induce paralysis, pain, and other effects. Venom from a small number of caterpillars (*Lonomia* sp.), assassin bugs (*Pristhesancus plagipennis*, *Peirates turpis*), giant water bug (*Lethocerus dintinctifemur*), and robber flies (*Eutolmus rufibarbis*, *Machimus arthriticus*) have been recently investigated using combined transcriptomic/proteomic approaches, which revealed that all contain both peptides and larger proteins. The defensive venom of *Lonomia* sp. venom contains enzymes and lipocalins that produce drastic coagulopathies that can be fatal to large mammals such as humans. Venoms used for prey capture by Heteroptera and Asilidae contain both linear and disulfide-rich peptides, some of which have been demonstrated to be neurotoxic. Some insect toxins are derived from protein families that have been convergently recruited into the venoms of insects and other animal groups, including ICK peptides, CRiSPs, and enzymes such as proteases and hyaluronidases. In contrast, the protein families of unknown function that dominate the venoms of Heteroptera and Asilidae suggest unique neofunctionalisation events.

The high number of neglected groups of venomous insects, and the speciose nature of many of these, suggests they are likely to be rich sources of new pharmacological agents for biotechnology and medicine. However, to capitalise on this opportunity, further studies are required to elucidate the basic biology of venom use, including the glandular source of bioactive venom, in many groups. The hyperdiversity of venom use among insects, combined with recent technological advances, is likely to deliver a large amount of information on the structure and function of novel venom toxins in the near future.

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Table 1: Insect groups that have independently evolved venom use.

| Lineage | Common name | Taxonomy | Stage | Venom glands | Injection apparatus | Prey capture | Defense | Composition | References |
|---|---|---|---|---|---|---|---|---|---|
| 1 | True bugs | Hemiptera: Heteroptera | All | Salivary glands | Maxillary stylets | | | Proteases, ICK, pore-forming, unknown | Walker et al. (2017; 2018a; 2018b), Edwards (1961) |
| 2 | Soldier aphids | Hemiptera: Hormaphididae and Pemphigidae | Sterile clones | Alimentary canal? | Maxillary stylets | | | Cathepsin B | Kurosu and Aoki (1988a), Stern and Foster (1996), Kutsukake et al. (2004) |
| 3 | Wasps, ants, and bees | Hymenoptera: Apocrita | Adult female | Modified sex accessory | Modified ovipositor | | | Peptides especially helical, enzymes, alkaloids | Piek (1986), Robinson et al. (2018), Konno et al. (2016), Touchard et al. (2016a) |
| 4 | Antlions and allies | Neuroptera | Larvae | Maxillary gland or alimentary canal | Maxillae/ mandibles | | | ? | Canard (2001), Henry (1977), Gaumont (1976) |
| 5 | Larval fireflies and allies | Coleoptera: Lampyridae | Larvae | Alimentary canal? | Mandibles | | | ? | Fabre (1924), Williams (1917), Hess (1920) |
| 6 | Larval rove, water beetles and allies | Coleoptera: Staphylinoidea and Hydrophiloidea | Larvae | Alimentary canal? | Mandibles | | | ? | Balduf (1935), Heymons (1927), Richmond (1920) |
| 7 | Larval diving, ground beetles and allies | Coleoptera: Adephaga | Larvae | Alimentary canal? | Mandibles | | | ? | Balduf (1935), Pennak (1953) |
| 8 | Scorpion beetle | Coleoptera: Cerambycidae: *Onychocerus albitarsis* | Adult | Antennal glands | Terminal antennal segment | | | ? | Berkov et al. (2008) |
| 9 | Buck moth caterpillars | Lepidoptera: Saturniidae: Hemileucinae | Larvae | Secretory cells in spines | Cuticular spines | | | Hemolin-like, lipocalin, enzymes, peptides | Alvarez-Flores et al. (2010; 2011), Veiga (2005), Maggi and Faulhaber (2015) |
| 10 | Flannel and cup moth caterpillars | Lepidoptera: Zygaenoidea | Larvae | Secretory cells in spines | Cuticular spines | | | ? | Kawamoto (1978b), Murphy et al. (2010) |
| 11 | Robber flies | Diptera: Asilidae | Adult | Thoracic glands | Hypopharynx | | | ICK, unknown | Drukewitz et al. (2018), Kahan (1964) |
| 12 | Larval horse flies and allies | Diptera: Tabanomorpha | Larvae | Mandibular glands | Mandibles | | | ? | Teskey (1969), (Otsuru and Ogawa, 1959) |
| 13 | Snail- and slug- fly larvae | Diptera: Sciomyzidae | Larvae | Salivary glands? | Mandibles | | | ? | Trelka et al. (1970; 1977), Berg and Knutson (1978) |
| 14 | Aphid midge | Diptera: Cecidomyiidae: *Aphidoletes aphidimiza* | Larvae | Salivary glands? | Mandibles | | | ? | Mayr (1974) |

**Figure 1: Multiple evolutionary origins of venom use among insects.** Orders with members that inject venom into animals for prey capture (marked with a knife and fork) or predator deterrence (marked with a shield) are highlighted in grey. An example of each of the 14 proposed venomous lineages is shown on the right. Lineage 1, true bugs (Heteroptera). Lineage 2, soldier aphids (sterile defensive caste of some Pemphigidae and Hormaphididae). Lineage 3, wasps, ants and bees (Apocrita); photo by Alejandro Santillana. Lineage 4, antlions and allies (Neuroptera); photo by Joseph Berger. Lineage 5, larvae of ground, diving beetles and allies (Adephaga). Lineage 6, larvae of rove, water beetles and allies (Staphylinoidea and Hydrophiloidea); photo Nikolai Vladirimov. Lineage 7, larvae of fireflies and allies (Lampyridae). Lineage 8, scorpion beetle (*Onychocerus albitarsis*); photo Alicia M. Hodson (Lingafelter et al., 2017). Lineage 9, buck moth caterpillars (Hemileucinae). Lineage 10, flannel and cup moth caterpillars (Zygaenoidea). Lineage 11, robber flies (Asilidae). Lineage 12, larvae of horse flies and allies (Tabanomorpha); photo Jim Moore. Lineage 13, snail- and slug-killing fly larvae (Sciomyzidae); photo Andre DeLorme. Lineage 14, aphid midge (*Aphidoletes aphidimyza*), photo by Erik Maurer.

**Figure 2: Insect venom glands.** (**A**) 3D reconstruction from MRI of the venom gland system of the assassin bug *Pristhesancus plagipennis* (Walker et al., 2018b) showing dual glands for venom secretion, the anterior main gland, vg-amg (dark blue) and posterior main gland, vg-pmg (red). Accessory gland, ag (light blue); alimentary canal, ac (yellow); venom pump, vp (pink). (**B**) 3D reconstruction from MRI of the venom gland system of the robber fly *Eutolmus rufibarbis* (Drukewitz et al., 2018). Venom glands (thoracic glands), vg (pink); pp, pharyngeal pump; lb, labial gland; m, musculature. See original publication for enlargements of insets D, E. (**C**) Morphology of venom apparatus of an ant, *Rhytidoponera* sp. (Robertson, 1968). Venom glands, vg; venom reservoir, vr; dg, Dufour's gland; sb, sting bulb with valves; st, sting.

**Figure 3: Insect organs used for venom injection.** (**A**–**C**) Stylets of the giant water bug *Belostoma lutarium* (Swart and Felgenhauer, 2003). A, stylet bundle emerging from the sheath-like labium; scale bar 250 μm. (**B**) Mandibular stylet; bar 100 μm. (**C**) maxillary stylet; bar 50 μm. (**D**, **E**) Venom spines of the caterpillar *Lonomia obliqua* (Quintana et al., 2017). (**D**) Spine clusters or scoli; bar 2 mm. (**E**) Tips of spines; bar 100 μm (**E**). (**F**, **G**) Hymenopteran stingers (Baumann et al., 2018). (**F**) Yellowjacket *Vespula loctuosa*; bar 160 μm. (**G**) Ant *Paraponera clavata*; bar 150 μm. (**H**, **I**) Hypopharynx of the giant robber fly *Dolopus genitalis*. (**H**) Lateral view; bar 500 μm. (**I**) close up of ventral surface showing pore; bar 200 μm. (**J**–**L**) modified antenna of the longicorn beetle *Onychocerus albitarsis* (Berkov et al., 2008). (**J**) Terminal three segments; bar 1 mm. (**K**) Stinging tip; bar 100 μm. (**L**) Pores on tip; bar 20 μm. (**M**), Pincer-like jaws of the antlion *Palpares inclemens* (Mansell, 1999). The maxilla has been lifted from the mandible on the left side. Jaw length is 8 mm.

**Figure 4: 3D structure of hymenopteran venom peptides.** (**A**) Melittin, a membrane-disrupting peptide from the honeybee *Apis mellifera* (Perekalin Dmitry et al., 2015). (**B**) Ectatomin-1, a homodimeric peptide that disrupts membranes and inhibits calcium channels, from the ant *Ectatomma*

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**Figure 5: Neuropteran maxillary gland.** Cross-section of the basal part of the jaw of a lacewing larva, comprising the mandible (md) and maxilla (mx) that interlock to form the food canal (fc) used to take up liquid food from prey into the gut. One possible source of toxins that paralyse prey is within the maxilla, here labelled venom gland (vg) and venom channel (vc) following Canard (2001).

**Figure 6: Phylogenetic diversity of venomous Coleoptera.** Highly simplified phylogeny following Hunt et al. (2007). Major taxa containing venomous species are highlighted in grey. Symbols are as for Fig. 1.
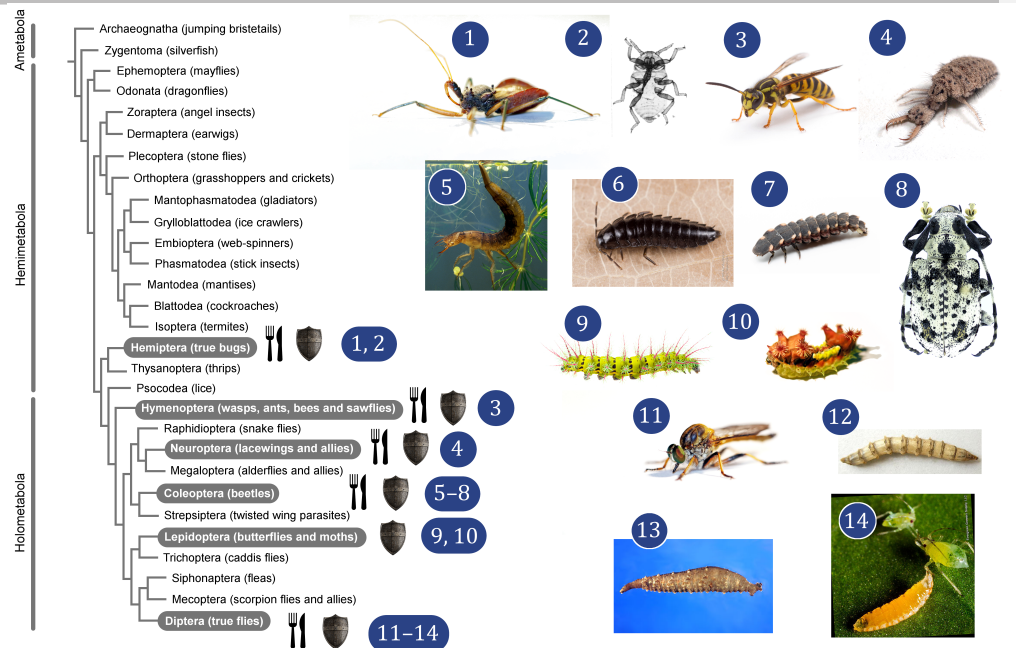
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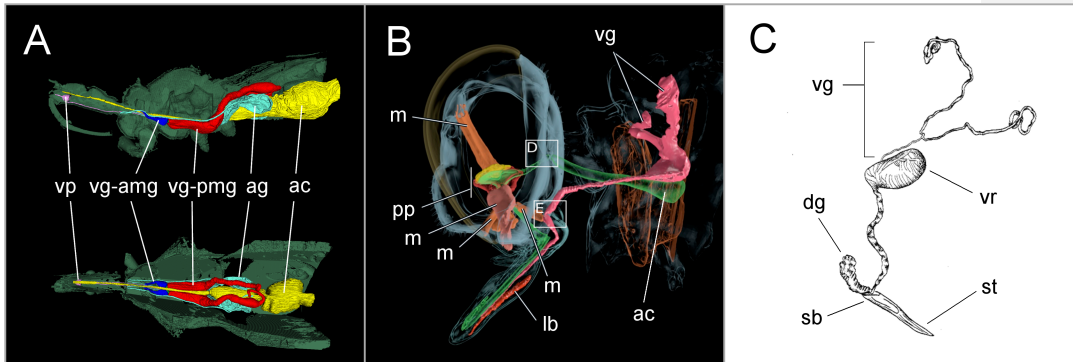
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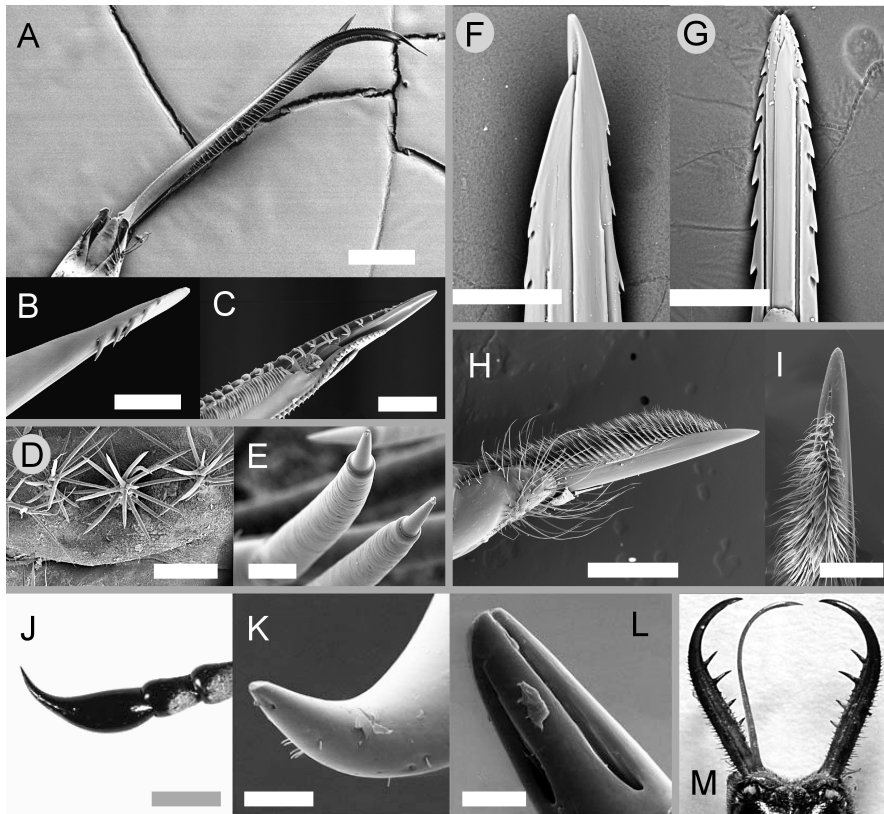
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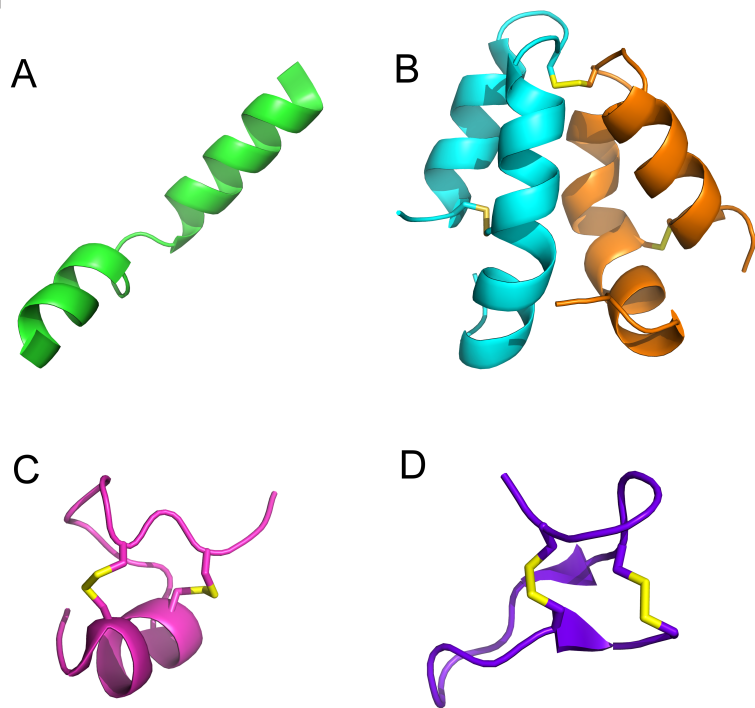**Deleted:** Lthree

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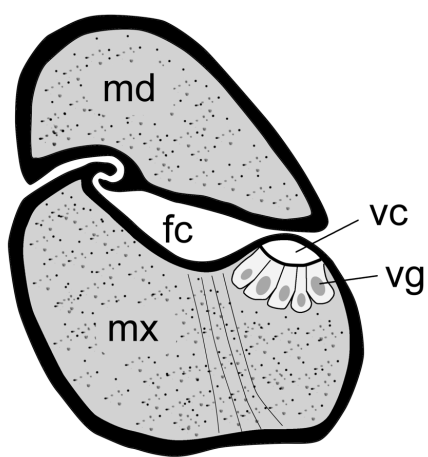
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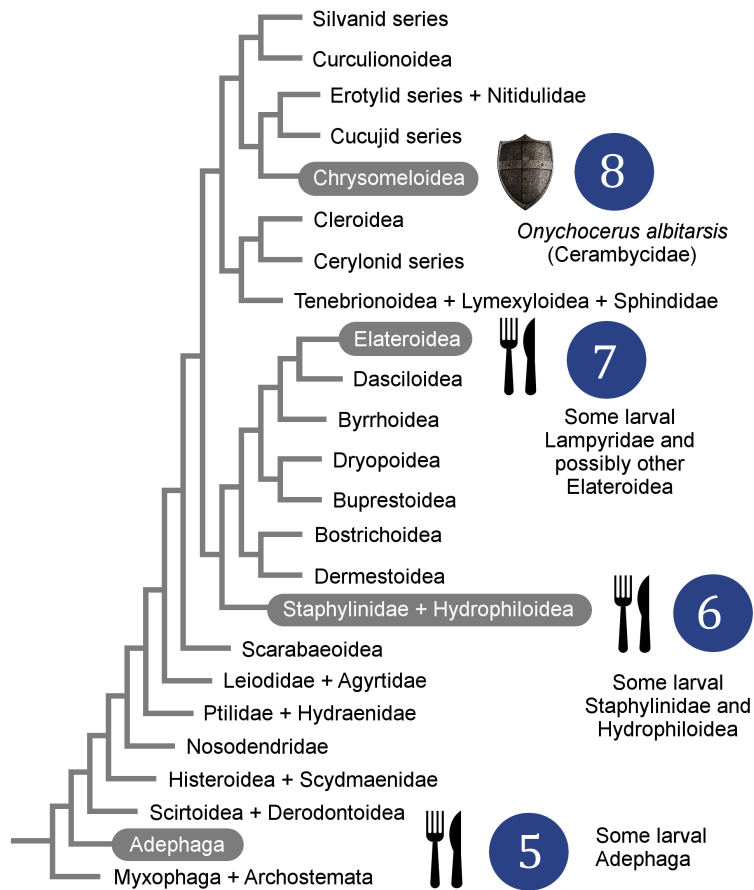
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Highlights

- Literature review suggests that venom use has evolved 14 times among the insects, the most diverse class of animals.
- The biology of venom use reveals structural and functional convergence in the physiological and molecular basis of envenomation between distinct groups of venomous insects.
- The insects represent an enormous and mostly untapped source of new toxins, including peptides, enzymes, pore-forming proteins and alkaloids.