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Adaptations and counter-adaptations in *Drosophila* host–parasitoid interactions: advances in the molecular mechanisms

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Both hosts and parasitoids evolved a diverse array of traits and strategies for their antagonistic interactions, affecting their chances of encounter, attack and survival after parasitoid attack. This review summarizes the recent progress that has been made in elucidating the molecular mechanisms of these adaptations and counter-adaptations in various *Drosophila* host–parasitoid interactions. For the hosts, it focuses on the neurobiological and genetic control of strategies in *Drosophila* adults and larvae of avoidance or escape behaviours upon sensing the parasitoids, and the immunological defences involving diverse classes of haemocytes. For the parasitoids, it highlights their behavioural strategies in host finding, as well as the rich variety of venom components that evolved and were partially acquired through horizontal gene transfer. Recent studies revealed the mechanisms by which these venom components manipulate their parasitized hosts in exhibiting escape behaviour to avoid superparasitism, and their counter-strategies to evade or obstruct the hosts' immunological defences.

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Introduction
An estimated 10% of all insects are parasitoids, and this life style evolved repeatedly in multiple insect orders [1]. Parasitoids lay their eggs in or on host arthropods, and their larvae destructively feed on the body of this host, killing it in the process [2]. Virtually all insect species are host to one or multiple parasitoid species, and it is therefore argued that Hymenopteran parasitoid diversity could surpass the beetles as the most species-rich insect order [3]. Parasitoids form a major mortality factor in the ecology of many insects, and hence impose a very strong

selection pressure on their hosts to evolve defensive strategies, including behaviours to avoid parasitoid encounters and attack, and immunological defences. This, in turn, imposes strong selection on parasitoids to overcome these host defences, as typically only the parasitoid or the host can survive the interaction. The strong, antagonistic, and dynamic co-evolution between hosts and parasitoid has led to a tremendous diversity in adaptations and counter-adaptations in host–parasitoid interactions [2,4]. In recent years, substantial progress has been made in elucidating the molecular mechanisms of such adaptations and counter-adaptations, particularly in various *Drosophila* host–parasitoid interactions. In this review, I will highlight some of the advances that have been made, to illustrate the diversity and complexity of evolving traits in host-parasitoid interactions (Figure 1).

Drosophila species are host to both larval and pupal parasitoid species [5]. In recent years, the diversity of parasitoid species that attack *Drosophila* have received renewed interest, in particular to investigate the potential of *Drosophila* parasitoids to control the population of an invasive and highly destructive pest, *Drosophila suzukii* that is rapidly spreading worldwide [6–8]. Moreover, the model species *Drosophila melanogaster* is being used extensively to study behavioural genetics, immune defences and neurobiology, which form important components of the adaptations against parasitoids. Finally, the whole-genome sequences of multiple *Drosophila* species are available [9–11], while whole-genome sequences of various *Drosophila* parasitoids are now also being published [12–17]. This makes the system ideally suited to gain mechanistic insight in the evolution of adaptations and counter-adaptations in host–parasitoid interactions.

Parasitoids' searching and hosts' avoidance strategies
Hosts are often located in small, confined parts of the environment, and are under selection to remain hidden from their parasitoids. The range of breeding sites for hosts may differ among geographic locations, seasons and habitats. Consequently, parasitoids evolved flexible searching strategies for long-distance localisation of sites that potentially contain their hosts. This includes an innate attraction to the volatiles of fermenting or decomposing organic substrates (i.e. the typical breeding site for most *Drosophila* species), as reported in both larval parasitoids of the genus *Leptopilina* and the pupal parasitoid

Figure 1
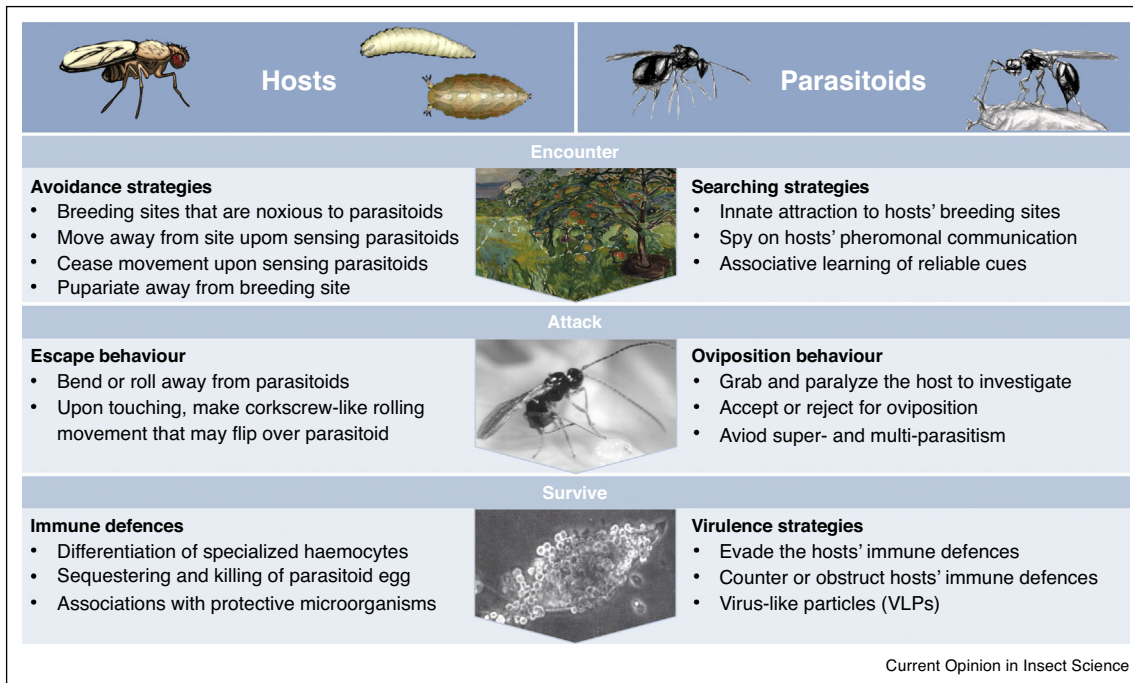
Adaptations and counter-adaptations in *Drosophila* host-parasitoid interactions.
The antagonistic host–parasitoid interaction can be subdivided into three stages, for which both hosts and parasitoids evolved various traits and strategies: encounter, attack and survival. Firstly, the parasitoids seeks to find the hosts that are typically confined to small, ephemeral breeding sites. Hosts employ various strategies to avoid detection from searching parasitoids, while parasitoids exploit an array of cues that may direct them to host breeding sites. Upon sensing the parasitoids' presence, hosts exhibit various behaviours to escape from the parasitoid attack. The parasitoids in turn evolved behaviours and morphological adaptations that aid them in localizing and parasitizing their hosts. After parasitization, only one of the two parties can survive, and both hosts and the parasitoids employ various strategies to enhance their own chances of survival. This review highlights some of the recent mechanistic insights that we gained in the evolution of these diverse adaptations and counter-adaptations.

Trichopria cf. *drosophilidae* [18,19]. Female *Leptopilina heterotoma* also spy on the pheromonal communication of adult *Drosophila* to localise larval feeding sites [20,21]. Additionally, many parasitoids can learn, linking particular environmental cues (e.g. odours, colours) to the presence of suitable host sites through associative memory formation as well as priming and sensitization of the sensory sytems. Especially on the topic of parasitoid learning, progress has been made on the neuronal pathways and mechanisms of short-term and long-term memory formation [see Ref. [22] for a recent review]. This review highlights the mechanisms and dynamic nature of memory formation, including the ability of parasitoids to constantly update these memories to tailor their behavioural responses to those cues that reliably predict suitable host locations in variable environments.

To avoid searching parasitoids, adult *Drosophila* may lay their eggs on breeding substrates that are unsuitable or even toxic to parasitoids, for example, on ripe noni fruits with high octanoic acid levels, or substrates with high concentration of atropine or ethanol [23–25]. Additionally, both adults and larvae can sense the presence of

volatiles emanating from *Leptopilina* parasitoids through a highly conserved olfactory receptor (Or49a), triggering a rapid moving away from the site, and causing females to refrain from egg-laying [26–28]. Female *Drosophila* can also visually perceive the presence of several (relevant) larval and pupal parasitoid species, which induced a, yet unexplained, acceleration of receptiveness to mating, accompanied by a strong increase in the expression of the gene *IBIN* [29]. In larvae of *Drosophila*, this *IBIN* gene was also induced in the fat body and haemocytes after parasitoid infection, leading to increased numbers of circulating haemocytes [30]. Whether the visual perception by adult females of parasitoids also boosted their offsprings' immune defences has yet to be established. It has been shown, though, that adult *Drosophila* can prime the immune system of their offspring by maternal provisioning of mRNA for pattern recognition receptors, upon perceiving the presence of parasitoids, which resulted in a more rapid induction of the larval cellular immune responses [31•]. *Drosophila* larvae can also sense sounds and vibrations that may indicate the presence of probing parasitoids, and behaviourally respond by either abruptly ceasing their movements (startle-freeze behaviour), or

rolling or fast-crawling away from the stimulus [27,32]. These behavioural responses require Cho neurons, and are mediated by TRP channels [32]. Furthermore, they can display similar behavioural responses after triggering of mechanosensory and nociceptive neurons, which may be activated by touch or stinging of the parasitoid. The neuronal circuitry for these behavioural responses involves projection and interneurons, which converge on a selective set of motor neurons in the nerve cord, called Goro neurons [27,32]. Hence, a whole suite of sensory modalities appears to be utilized by *Drosophila* to avoid parasitoid encounters.

Parasitoid attack and host escape behaviour

Once the parasitoid has found a host, it inserts it ovipositor into the host. This ovipositor is equipped with sensory organs, enabling the parasitoid to assess the suitability of the host for oviposition [33]. Moreover, it may contain morphological features for bending, steering and grabbing hold of the moving larvae [34,35]. Once a larva is pricked, it can be immobilized with a clip-like structure on the ovipositor [35], or by injection of substances that induce short-term paralysis [36]. This allows the parasitoid to assess the quality and suitability of the host, and to decide whether to accept or reject the host for oviposition. Larval and pupal parasitoids of *Drosophila* are typically solitary, injecting a single egg in their host. Parasitoids often can recognize whether a host has already been parasitized, and may decide to reject the host for oviposition to avoid 'superparasitism' (i.e. multiple parasitoids of the same species parasitizing the same host) or 'multiparisitism' (i. e. multiple parasitoids of different species parasitizing the same host) [37]. Interestingly, parasitoids can also manipulate the subsequent behaviour of the parasitized larvae by injecting RhoGAP domain-containing proteins. In a comprehensive analysis, including behavioural observations, transcriptomics, functional genetics and evolutionary genomics, it was shown that the injection of these proteins induced an escape behaviour in parasitized hosts that resulted in lower rates of superparasitism [38•].

Hosts can perceive touching and pain through nociception sensory neurons on their cuticle. The mechanosensory detection of touching induces a stereotypical bending away and corkscrew-like rolling movement [39], seemingly similar to their response to sound [27]. Several genes and neuronal subpopulations involved in nociception and behavioural responses have been characterized in *D. melanogaster* larvae [39–44]. These innate behaviours are elicited by the penetration of the larval cuticle by the parasitoids ovipositor. As the rolling movement of the larvae can result in female wasps being flipped over and the larva being freed from the ovipositor, this is considered an host adaptation to avoid or escape parasitoid attack [39].

Immunological host defences

Once a host is being accepted for oviposition by a parasitoid, its' only chance of survival is to kill the parasitoid before it is being killed itself. Insects possess potent immunological defences against a variety of pathogens, parasites and parasitoids. Immunological defences against larval parasitoids have been studied more extensively than against pupal parasitoids, but see [45]. The genes that are differentially expressed in larvae during the immune response after parasitoid attack have been characterized against various larval parasitoid species [46,47]. These responses include the timed regulation of various immunity genes, including pattern recognition receptors, members of the Toll, Imd and Jak/STAT immunity signal transduction pathways, several proteases and effector molecules [46,47]. Additionally, the infestation with parasitoids may alter the expression of metabolic pathways in the gut of the host larvae [48]. This may be associated with the energetic costs of launching an immune response [46,49], or it may constitute an active manipulation of parasitoids to enhance the nutritional quality of their host [48].

The larval immune response to parasitoids typically involves the proliferation and differentiation of specialized haemocytes (i.e. the insect blood cells) that sequester and kill the parasitoid egg before it hatches [50]. The specialized haemocytes for encapsulating parasitoid eggs are mostly absent or rare in uninfected larval hosts, but their proliferation and differentiation are induced upon parasitoid infection. For *D. melanogaster*, the differentiation into different types of blood cells has been carefully described, both with flow cytometry approaches [50] and single-cell transcriptomics [51–55]. These studies reveal a near-continuous range of cell types that progress towards various end-points of differentiation. The high-resolution expression profiles in single-cell analyses exposed also new subpopulations of cells that were not previously recognized. To what extent these form separate entities with individualized functionalities remains to be investigated.

The proliferation and differentiation of haemocytes occurs in the larval lymph glands, circulating pro-haemocytes and in sessile clusters of haemocytes that reside in subepithelial pockets [50,56,57]. These sessile clusters can be induced to release the haemocytes by activation of multidendritic and Cho sensory neurons that innervate with these pockets, through *Activin-β/dSmad2* signalling. This activation induces the neurons to produce *Activin-β* ligands that bind to haemocyte receptors of the *dSmad2* pathway and induces the haemocytes to proliferate [58]. The genetic and regulatory control of the proliferation and differentiation of haemocytes, during each life stage and in different anatomical compartments, is being investigated in great detail in *D. melanogaster* [recently reviewed in Ref. 59•].

A diversity of haemocytes evolved for parasitoid defences in *Drosophila*, with several types of haemocytes being restricted to specific clades of the *Drosophila* phylogeny [60–64] (Figure 2). The evolutionary origin of distinct haemocyte classes in *Drosophila* immune defences remains largely unresolved. Classification of insect haemocytes is complicated, as cell types with similar functional roles can differ markedly in morphology and stereotypic characteristics [65,66]. For example, lamellocytes (in the *melanogaster* subgroup) and pseudopodocytes (in the *obscura* subgroup) function similarly in the encapsulation of parasitoid eggs, which is accompanied by melanisation of the sequestered egg in both clades. The pseudopodocytes resemble lamellocytes in morphology, yet are distinct in having phagocytotic capacity, a somewhat smaller size, round to worm-like cell shapes, and irregular plasma membrane surfaces systematically covered by long, thin pseudopodia [61]. In contrast, the multinucleated giant cells in the *ananassae* subgroup, and the mononucleated nematocytes in *Zaprionus indianus*, a member of the subgenus *Drosophila*, do not remotely resemble the lamellocytes and pseudopodocytes, and function entirely differently in immune responses, without melanisation of the sequestered parasitoid egg [62,63]. Although lamellocyte-like cells and multinucleated giant cells have also been reported for other drosophilid lineages [63,67], they are largely absent in other clades. It is therefore unclear whether the similar classes of haemocytes are strictly homologous, or whether they arose through convergent evolution of novel classes of haemocytes.

In a comparative analysis among 11 sequenced *Drosophila* species, we showed that lamellocytes were restricted to a monophyletic clade (the *melanogaster* subgroup), although the genes required for the differentiation of the lamellocytes in *D. melanogaster* were strongly conserved among all 11 sequenced species. Eleven genes that were induced by parasitoid attack arose around the time of lamellocyte acquisition, through gene duplications and divergence [64]. One species within this *melanogaster* subgroup, *Drosophila sechellia*, secondarily lost its ability to resist parasitoids. Its transcriptional response to parasitoid attack was largely altered [68] and several of the 11 novel genes showed loss-of-function mutations [64]. This would suggest that lamellocytes are, in fact, the product of an evolutionary innovation that emerged in the *melanogaster* subgroup by co-option of existing haemocyte differentiation pathways and the integration of novel components in the gene regulatory network [64]. To fully resolve the evolutionary origin of the various classes of specialized haemocytes will require a careful comparison of the genetic pathways that regulate their differentiation.

When populations of *Drosophila* are exposed to high levels of parasitoid attack, they rapidly evolve increased parasitoid resistance, both in experimental evolution settings and in nature [69–71]. This selection for increased resistance resulted in increased numbers of circulating haemocytes [71,72,73], changes in gene expression during early development and in blood cell differentiation [52,72], and changes in allele frequencies within narrow genomic regions [74]. The haematopoietic responses to parasitoid attack can vary rather dramatically between host populations and genotypes [75,76], and this also interacts with the parasitoids' genotype [76]. Moreover, the physiological basis and developmental mechanisms underlying the increase in circulating haemocytes after strong selection for parasitoid resistance differ among *Drosophila* species: Species either increased the production of the total numbers of haemocytes, or they moved sessile haemocytes into circulation. These increased haemocyte loads are costly in terms of larval competitive ability [71].

In addition to these endogenous host immune defences, several *Drosophila* species also formed associations with microorganisms that can provide protection or enhanced resistance to parasitoids [77]. The extent to which these endosymbionts may offer protection against parasitoids varies between parasitoid genotypes, and may also be modulated by environmental factors (e.g. ethanol concentrations in the breeding site) [78]. One mechanism by which the microorganism can provide protection is by depletion of lipids from the haemolymph of the pupal host, which inhibits the parasitoids growth by means of metabolic competition, without inducing the cellular immune response in the host [79]. This could be especially detrimental for larval parasitoid development due to the evolutionary trait loss of *de novo* lipogenesis in many parasitoid species, including species of *Asobara* and *Leptopilina*. Therefore, parasitoid larvae are expected to mostly rely on consuming host lipids, and in their early stages of development acquire these mostly from host haemolymph [80]. In addition, the endosymbionts can produce toxins that impede parasitoid development, such as a family of ribosome-inactivating proteins (RIP), encoded by *Spiroplasma*, that strongly affect early development of *L. heterotoma* and *Leptopilina boulardi* but not of *Pachycrepoideus vindemmiae* [81].

Parasitoid virulence factors

Parasitoids evolved various strategies to evade hosts immune defences. In some species of *Asobara* parasitoids, the eggs are coated with long velcro-like appendages that make the eggs 'sticky' [82,83]. Also eggs of some *Leptopilina* parasitoids can show stickiness, but this seems to be related to a venom protein that is injected into the host during oviposition, containing a putative mucin-binding domain [84•]. In both cases, the adhesive attachments of the parasitoid eggs to host tissues impedes the adherence of host haemocytes and melanisation of the parasitoid egg, and this incomplete sequestering and melanisation

Figure 2
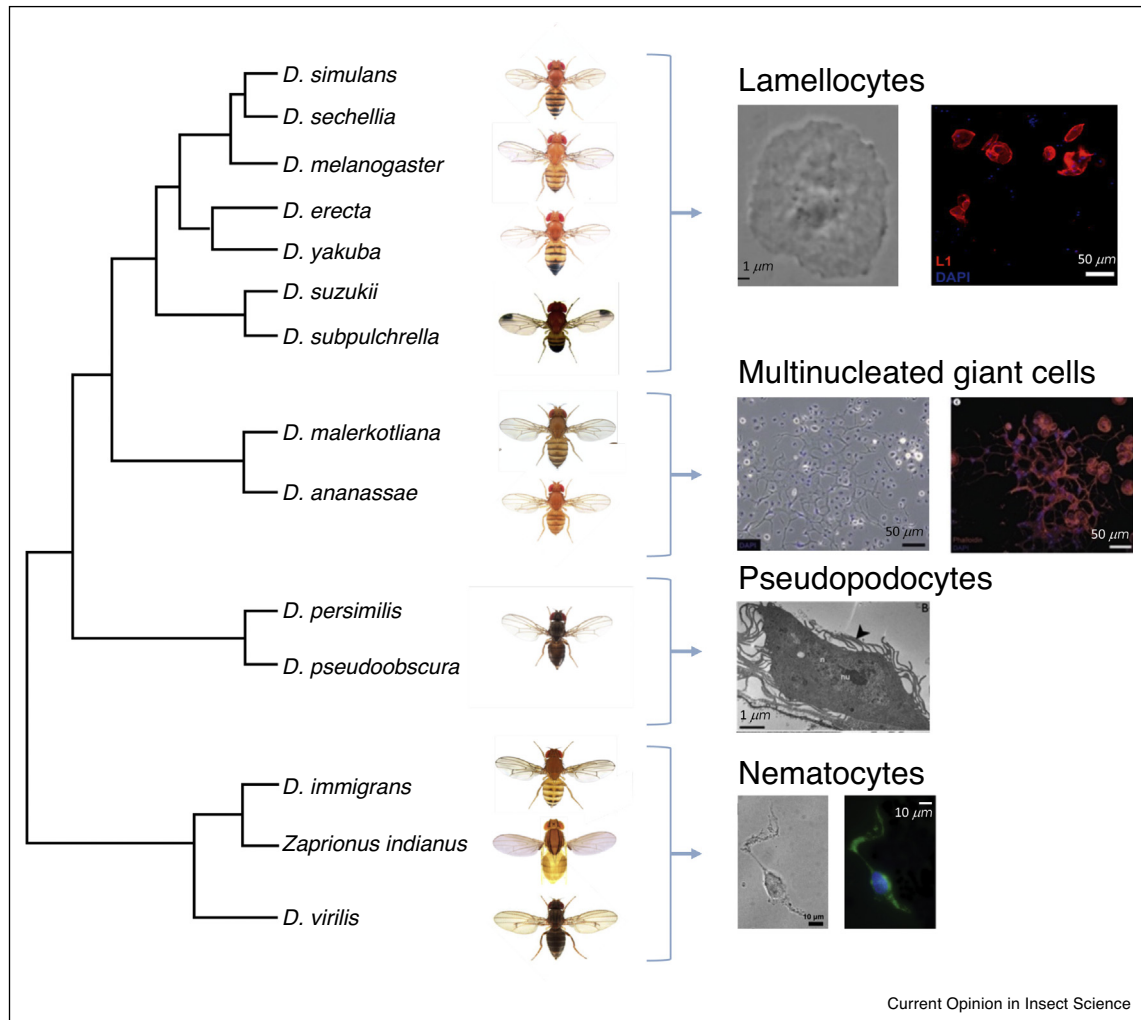
Specialized types of haemocytes that evolved for immune responses against parasitoids in the *Drosophila* phylogeny.
Lamellocytes in the *melanogaster* subgroup are large (25−40 μm), extremely flat haemocytes with adhesive properties rarely found in healthy larvae, but that differentiate in large numbers from ∼20 hours after parasitoid infection [50,60]. Multinucleated giant cells evolved in the *ananassae* subgroup; these haemocytes vary in size from 40 to several hundred micrometers, and consist of syncytia without separating cell membranes, but with complex membrane extensions. They fully develop 48−96 hours after parasitoid infection, and sequester and kill parasitoid eggs without melanisation [62]. Pseudopodocytes in the *obscura* subgroup resemble lamellocytes in morphology and functioning, yet are distinct in having phagocytotic capacity, a somewhat smaller size (15−40 μm), round to worm-like cell shapes, and irregular plasma membrane surfaces systematically covered by long, thin pseudopodia (arrowhead) [61]. Nematocytes in *Zaprionus indianus*, a member of a clade embedded in the genus *Drosophila*, are large spindly (fusiform), mononuclear haemocytes with long filipodia extending from the dominant cell axis, that increase in number and in length after immune challenge, and that sequester parasitoid eggs (mostly) without melanisation [63]. Pictures of haemocytes reproduced from [61–63] with permission; *Drosophila* pictures from http://gompel.org/drosophilidae.

results in parasitoid eggs that can hatch and evade the hosts immune defences [82,83,84•].

Parasitoids can also actively counter or obstruct different components of the host immune responses. The venoms that parasitoids inject during oviposition have multifaceted immunomodulatory properties, and the diverse cocktails of venoms have been characterized with transcriptomic and proteomic approaches [12,84•,85–87]. The mechanisms by which individual venom components can suppress host immune responses include factors that antagonize the hosts' intracellular calcium signalling to stop haemocyte differentiation, serpins that inhibit serine protease cascades and melanisation, toxins that inactivate hosts' RhoGAPs that are required for proper lamellocyte functioning, and proteins that cause lysis of the hosts' lymph gland or destroy the fully differentiated haemocytes [60,84•,88–92]. Experimental evolution studies

showed that the venom composition can rapidly diverge, within a few generations, when the parasitoids are exposed to different strains of the same host species [93].

Bioinformatic analyses revealed the dynamic evolution, as well as the diverse origins of the genes encoding for these venom components, including gene duplications, horizontal gene transfer (from protozoans, viruses and bacteria), co-option and multi-functionalization [12,16,84•,94,95]. Several *Drosophila* parasitoid species of the *Leptopilina* genus have been long known to employ virus-like particles (VLPs) that may aid them in obstructing the hosts' immune defences. The biotic nature of these VLPs is still under discussion, with some studies proposing that these VLPs are the result of an endogenization event of a virus [15,16], and others arguing that the VLPs are non-viral and more similar to extracellular vesicles [12,95]. A recent analysis traced the putative endogenization event of a virus that occurred in a species that is ancestral to all *Leptopilina* species, but after its divergence from *Ganaspis* [16]. Close extant relatives of this virus have been shown to manipulate the parasitoids behaviour and induce increased superparasitism, which could be beneficial for their own transmission to other parasitoids [15]. In contrast, proteomic studies revealed that several VLPs that are unique to *L. heterotoma* lack viral coat proteins, and possess properties that would suggest a mixed origin involving both eukaryotic microvesicles and bacterial surface secretion systems rather than viral origins [12,95]. These extracellular vesicles (EVs) of *L. heterotoma* have a distinctly different morphology and functioning in suppressing the hosts' immune defences, compared to the VLPs of *L. boulardi*, entering the lymph glands and causing their disintegration which largely prevents lamellocyte differentiation [96], as well as entering the haemocytes to damage or lyse them [95]. These studies indicate that multiple, but different virulence strategies evolved among closely related parasitoids infecting the same hosts.

Conclusion

Parasitoid–host interactions are characterized by dynamic and rapid co-evolution. To gain insight into the molecular mechanisms of evolution for these traits, major advances are now being made in elucidating how these traits are regulated and coordinated by multiple genes and neuronal activity. The advances that are being made rely partially on state-of-the-art functional genetics and genomics approaches. These approaches allow for the unbiased assessment of the diversity of genes and proteins involved in these interactions, followed by extensive functional characterization of specific putative elements. It also allows for a more extensive integration of neuronal biology in the behavioural and physiological strategies, as well as the regulatory control of immune defenses. *D. melanogaster* is ideally suited for this, as the knowledgebase and molecular toolkit in this model organism are ever

expanding, and a recently published 'connectome' of the neuronal circuits in the brain will aid in resolving the individual functionalities of all 100 000 neurons in behavioural and physiological control [97]. Finally, the increased availability of genome sequences for both hosts and parasitoids are fueling a wider array of bioinformatics analyses and evolutionary genomics to retrace the evolutionary origin of adaptations and counter-adaptations in host–parasitoid interactions. These comparative approaches are going to be especially valuable for resolving the genetic architecture of the fast evolving traits that arise and diversify in this continuous arms race between hosts and parasitoids. It may also aid in resolving some of the major open questions in the research field, concerning the origin and evolution of novel classes of host haemocytes and parasitoid venoms and virulence factors.

Conflict of interest statement

Nothing declared.

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