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Using *Drosophila* to study the evolution of herbivory and diet specialization

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Herbivory evolved many times independently across the insect phylogeny, and its evolution is linked with increased rates of diversification. Plants present many barriers to potential herbivores, among them are the so-called secondary chemicals and other molecular defenses such as protease inhibitors that deter herbivores. To understand the mechanisms behind the emergence of herbivory and subsequent species radiations of insects driven largely by diet specialization, it is important to identify the molecular basis associated with these evolutionary transitions. However, most herbivore species lack the genomic information and genetic tools required to identify functionally important genes. The notable exception is the genus *Drosophila* in which herbivory evolved at least three times independently, and for which abundant genomic data are available. Furthermore, contained within the family Drosophilidae is *Drosophila melanogaster*, the first genetic model animal. Here, we provide a synthesis of the salient tools that the *D. melanogaster* system provides to identify functionally important genes required for herbivory and subsequent diet specialization across insects.

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Introduction

Insects are the most diverse clade of life in terms of named species, which is linked to the successful invasion of an array of trophic niches. An herbivorous lifestyle is also typically a parasitic one. It has evolved in only a third of insect orders, but when it has, its establishment has been followed by conspicuous species radiations [1]. These species radiations are likely to be caused, in part, by co-evolutionary interactions between insects and their host plants [2]. Major herbivorous radiations include the Lepidoptera, as well as weevils (Curculionidae) and leaf beetles (Chrysomelidae) within the Coleoptera. To understand the mechanisms behind such dramatic switches in trophic niche and subsequent specialization (i.e. determination of host range), and the origin of new species [3•], it is important to identify their molecular basis, that is, the genes and allelic variants involved in adaptation and speciation [4,5]. Such a reductionist approach will further aid in synthesizing a general framework for linking agents of natural selection arising from species interactions and their targets in the genome, and is essential for the development of management strategies for agricultural pests.

However, one issue with identifying ecologically relevant genes and allelic variants in insect herbivores is that considerable resources are required to develop the necessary molecular toolkit. This barrier can be particularly limiting for researchers in the fields of ecology and evolutionary biology, where the number of people studying any one herbivore is typically limited.

The 'classic' model insect *Drosophila melanogaster* is not an herbivore itself, and definitive proof of the ecological relevance of genes and allelic variants can only be gained from experiments in the herbivorous insects themselves [6]. Nevertheless, this model system provides a number of advantages that could facilitate the characterization of the molecular basis of insect herbivore responses to plant secondary chemicals as we will describe here. In particular, gene products that are receptors or targets of plant secondary chemicals or are involved in detoxification and the regulation thereof should be readily identifiable.

There are three major advantages of *D. melanogaster* as a model system. First, the large academic research community using this model has assembled a comprehensive molecular toolkit [7], which we will describe in more detail below. This toolkit can be accessed through collaboration with academic colleagues or through outsourcing to the private laboratories that provide services to the *D. melanogaster* community. Second, the medical research community has amassed a significant body of knowledge on the response of *D. melanogaster* to a range of plant secondary chemicals relevant to human health including isothiocyanates, cardiac glycosides and nicotine [8].

Third, *D. melanogaster* is ideally suited for use in artificial selection and experimental evolution experiments for reasons that will be outlined below.

In addition to being a tool to studying the evolution of herbivory and diet specialization generally, herbivory has evolved at least three times within the family Drosophilidae, and at least 25 times within the order Diptera [1]. Some of these transitions to herbivory occurred < 20 million years ago [4], whereas in lineages such as the weevils and Lepidoptera these transitions occurred > 100 million years before present. In those lineages the sands of time render it difficult to identify many of the evolutionary genetic changes associated with the evolution of herbivory [4]. This means that more compelling biological hypotheses on the evolution of herbivory at the molecular level can be tested in the family Drosophilidae, which is aided further by the close proximity to *D. melanogaster*. However, for lineages in which herbivory evolved earlier, taking advantage of genetic assays in *D. melanogaster* it should still be possible to identify the salient evolutionary genetic changes involved in subsequent diet specialization, host shifts and co-speciation.

Here, we present approaches using *D. melanogaster* that could be used iteratively for the identification of candidate genes involved in the evolution of herbivory and diet specialization and subsequent functional characterization. In combination with laboratory and field studies of herbivorous insects themselves, these approaches form a key starting point to advance our understanding of some of the most important ecological interactions in terrestrial ecosystems.

Candidate gene identification

A key step towards understanding the molecular basis of herbivory and diet specialization is identifying candidate genes putatively underlying this phenotype. Candidate genes are those that show enough promise for linking genotype to phenotype such that functional studies are initiated.

Comparative genomics

The genus *Drosophila* with its rich genomic resources provides an ideal system for comparative genomics to systematically identify candidate genes linked to the evolution of herbivory and diet specialization [9–11]. Genomes of 22 *Drosophila* species are available on the database for *Drosophila* genetics and molecular biology FlyBase (http://www.flybase.org), and several more are in the process of being deposited including the genomes of two clades of herbivorous drosophilids: *Drosophila suzukii* and *Scaptomyza* spp. [10,11]. In addition, useful resources for comparative genomics have recently been developed for many herbivorous insects such as for the Lepidoptera (http://www.ensembl.lepbase.org). The use of a comparative approach has led to the identification of the molecular evolutionary basis of adaptations involved in herbivore tolerance to isothiocyanates and cardiac glycosides [12,13].

Transcriptome studies

Comprehensive tissue-specific whole-genome gene expression maps are available for *D. melanogaster* [14], including for (parts of) tissues that constitute important protective barriers against plant-derived toxins such as the blood–brain-barrier [15•] and the midgut [16•]. Other studies profiled genome-wide expression patterns following insect exposure to plant secondary chemicals such as isothiocyanates [4,17–19], or to reactive oxygen species [20]. These gene expression patterns, although specific to *D. melanogaster*, are often robust across millions of years of evolution and can be used to narrow the range of candidate genes identified through other means such as comparative genomics, mapping studies and genetic screens (see below).

Genome-wide association studies

Genome-wide association studies (GWAS) are a powerful approach to identify naturally occurring functional genetic variation underlying phenotypes of interest (such as susceptibility to plant toxins). GWAS rely on single-nucleotide polymorphisms (SNPs) in linkage disequilibrium (LD) (i.e., non-randomly co-inherited) with causative variants associated with phenotypic variation. The use of the recently established *D. melanogaster* Genetic Reference Panel (DGRP) [21] for GWAS has already led to the mapping of candidate genes involved in the fly's behavioral response to different odorants [22,23], and the fly's resistance to oxidative stress [24].

A pool-GWAS [25] approach allows identification of genomic regions associated with phenotypic variation in any species with a reasonably assembled genome (or a very close relative of one without). Regions not corresponding with phenotypic variation should assort randomly across the low and high pools but regions associated with the trait of interest, for example, lifespan in the presence of a plant toxin, will be statistically associated with one pool or another, based on allele frequencies. The theory behind a pool-GWAS is derived from a bulk segregant-type of quantitative genetics design, which requires analyses of progeny resulting from crosses of two divergent parental lines. Pool-GWAS studies do not require crosses and instead rely on natural recombination within the population of interest. The causative alleles are present in different genetic backgrounds and statistical tests allow identification of regions associated with the trait variation of interest. Population genetic analyses, including F_{st} between the two pools and Tajima's D across both will allow one to identify regions that may be targets of positive natural selection and associated with phenotypic variation [25]. These data can in turn inform future studies that may aim to functionally characterize these traits, for example.

In addition, artificial mapping populations such as the *Drosophila* Synthetic Population Resource (DSPR) [26] have been created, which have been used to identify candidate genes associated with nicotine resistance [27].

Artificial selection and experimental evolution

Next-generation sequencing has enabled the use of a 'evolve and resequence' strategy to identify candidate genes underlying adaptation to changing selection pressures such as the ingestion of novel plant toxins. Pool sequencing is conducted on an 'ancestral' starting population, which is then sub-divided over different treatment groups. Multiple insect population replicates are then reared in parallel under control and treatment selection regimes for multiple generations after which pools of insects from these different treatment groups are resequenced [28]. Comparison of genomic differences between the ancestral population and the population replicates evolved under control and treatment selection regimes then reveals genetic variants associated with adaptation to the novel selection pressure, which find their origins in standing genetic variation or less commonly as newly arising mutations [28].

Since LD breaks down rapidly in *Drosophila* spp., and flies generally have short generation times and large numbers of offspring, these insects are ideal for use in such experiments. The 'evolve and resequence' strategy has been successfully applied to find candidate genes involved in the adaptation to novel food sources and the odors that are associated with them [29,30]. However, large regions in LD with causative SNPs can complicate identifying the causative SNPs, a problem that may be ameliorated, in part, by initiating laboratory colonies with large numbers of founders from the wild.

Genome-wide mutant screens

In *D. melanogaster* large collections of transposon insertion lines [31], and RNAi lines [32] are available in which the function of individual genes is either knocked out through the insertion of a transposon in the promotor or within an exon of a gene, or the accumulation of gene translation products is prevented trough the targeted degradation of mRNA through RNAi. The loss-of-function transposon insertion mutant flies are available for nearly every gene for which its disruption does not result in lethality [31]. This allows screening of a family of genes for involvement in a phenotype of interest, as has been done for the ABC transporter genes [33].

When lethality is an issue, the tissue-specific expression of RNAi constructs is a potential solution to study gene function. Fly lines are available that have been transformed with the yeast transcriptional activator Gal4 under the control of tissue-specific promoters for a wide range of tissues. These flies can be crossed with gene-specific RNAi lines, which are also available for nearly all genes. The RNAi constructs in these lines are under the control of the UAS promoter, which is the target of Gal4 [34]. In the resulting offspring the RNAi construct is expressed in the Gal4-expressing tissue, leading to the destruction of mRNA from the gene of interest in the relevant tissue only. This clever approach has been used to screen Malpighian tubule-expressed organic anion transporter polypeptides for excreting the cardiac glycoside ouabain (a relative of digitoxin) from the insect [35].

Another useful resource is the Bloomington Deficiency Kit. This kit consists of nearly 500 *D. melanogaster* lines in which portions of chromosomes are systematically deleted. Crossing these fly lines with *D. melanogaster* strains that have mutations in genes whose products are targeted by plant secondary chemicals can identify genes that are indirectly under selective pressure from these chemicals. For example, ouabain exerts its toxicity by blocking the Na/K-ATPase, and the effects of ingesting this toxin and other cardiac glycosides can be mimicked by certain mutations in this ATPase [36,37]. Using the Deficiency Kit loci were identified that genetically interact with Na/K-ATPase function, which could lead to the discovery of novel genes involved in insect adaptation to dietary cardiac glycoside toxins [38].

Finally, the UAS-Gal4 system has been harnessed to create libraries of hundreds of fly lines that overexpress the open reading frame (ORF) of genes under the control of the UAS promoter, which has been termed the UAS-ORFeome [39]. This technique could be used to test tissue-specific overexpression mutants for gene families involved in the detoxification of plant toxins, and would form a useful complement with knock-out studies.

Functional tests of candidate genes

Once candidate genes and allelic variants have been identified, the stage is set to move beyond correlation/association and to show their molecular function and their consequences for the evolution of herbivory and diet specialization more definitively. *D. melanogaster* can be used to identify the involvement of candidate genes and allelic variants in the adaptation to host plant chemicals, in particular those involved in the detoxification and transport of plant toxins and the perception of plant volatiles. Below we outline several strategies that can be used for testing functionality of genes involved in herbivory.

Gene editing

The development of increasingly more efficient gene editing techniques could turn the use of gene editing and subsequent biological assays with transgenic animals into the 'gold standard' for establishing the functional importance of genetic changes associated with herbivory and diet specialization [6].

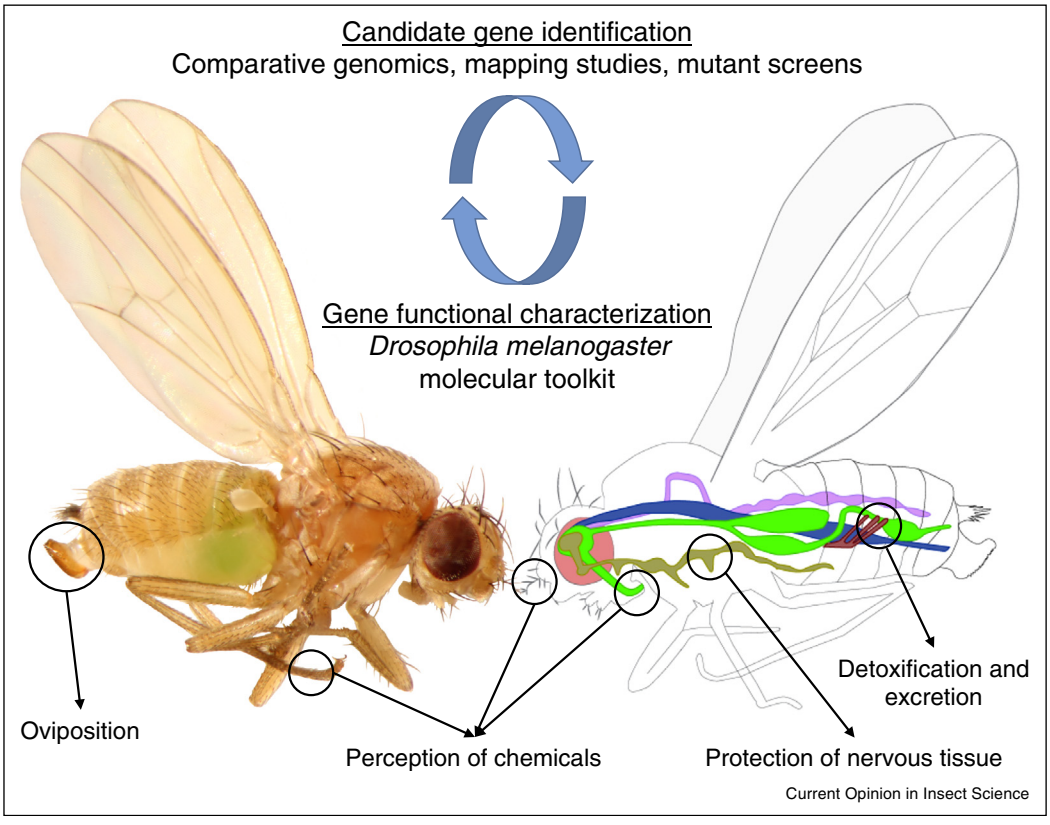
Several recent studies have used site-directed mutagenesis and transgenic expression to create cell lines expressing allelic variants of *D. melanogaster* genes that are involved in host plant adaptation in a range of insects. Examples include genes coding for detoxification enzymes such as glutathione *S*-transferases [12], and for Na/K-ATPase, the target of cardiac glycosides [13].

The establishment of gene editing techniques such as TALENs, zinc finger nucleases, and CRISPR/Cas9 for *D. melanogaster* will facilitate gene editing on a larger scale, with more flexibility and higher specificity regarding the exact modifications [40,41••]. The resources available for the use of the TALEN, zinc finger nuclease and CRISPR/Cas9 techniques have been reviewed previously [7], and we will not cover this further here. The CRISPR/Cas9 and zinc finger nuclease methods have been adapted for wild non-model herbivore species such as the Asian swallowtail (*Papilio xuthus*) [42••,43]. Yet, the facilities and tools available for *D. melanogaster* remain unparalleled. For example, its higher-quality genome assembly reduces the risk of encountering off-target effects because such sites can be more easily identified.

Tissue-specific gene expression

One valuable tissue-specific gene expression system germane to insect chemical ecologists, is the 'empty neuron' system [44], which can be used to test the function of olfactory and gustatory receptors (ORs and GRs). The 'empty neuron' fly line has been transformed with the yeast transcriptional activator (Gal4) and its target sequence (UAS), which induces the expression of a foreign OR or GR gene that is then expressed in a specific olfactory receptor neuron (ORN) subtype, in which the native OR, *Or22a*, is deleted (*ΔHalo*) [44,45]. Subsequently, one can conduct single sensillum recordings of

Figure 1



Comparative genomics between congeneric herbivores and between herbivores and their most closely related non-herbivorous relatives can be used to identify candidate genes involved in the evolution of herbivory and diet specialization. Other methods for identifying candidate genes could involve mutant screens and/or mapping studies. The roles of these candidate genes can be functionally verified using the *Drosophila melanogaster* molecular toolkit. These methods can be used in an iterative process to systematically identify adaptive roles for genes involved in several processes linked to herbivory such as oviposition, the perception of olfactory or gustatory signals, the detoxification and excretion of plant defense chemicals, the protection of nervous tissue. The figure shows the herbivore *Scaptomyza flava* in the family Drosophilidae as an example with on the left an adult female after the ingestion of leaf material (note the green-colored abdomen) and on the right a schematic representation of a subset of insect internal organs (green: digestive system, including in red the Malpighian tubules; brown: nervous system; blue: respiratory system; purple: circulatory system).
Image credit: Anna Nelson-Diettrich and Sophie Zaaijer.

the neuron expressing the foreign OR or GR, which can be used to assess the ability of ligands to activate the transgenic receptor [44,45]. Using this system, the *D. melanogaster* ORs have been screened for responsiveness to a wide range of odorants [46]. Conveniently, it is likely that any insect OR gene can be introduced into the empty neuron system [44]. However, evolutionary context is useful in the case of *Scaptomyza*, in which drosophilid-specific OR genes critical for detection of yeast volatiles have been lost [11].

A second set of heterologous gene expression systems is the transgenic expression of herbivore detoxification enzymes in whole flies and cell lines. Using these systems a cytochrome P450 enzyme of aphids adapted to a nicotine-containing diet was transgenically expressed in *D. melanogaster*. Transgenic flies showed reduced mortality when encountering this plant secondary chemical in their diet [47].

Behavioral assays

Numerous assays are available for *D. melanogaster*, which allow for the detailed observation of larval and adult fly behavior in response to various stimuli. When used in combination with the candidate gene identification and functional genetic techniques described above, these form a powerful approach to dissect the genetic basis of behavior.

A recent study compared the sensitivity of several assays that can be used to assess feeding in adult flies, the capillary feeder (CAFE) assay, food labeling with a radioactive tracer or colorimetric dye, and observations of proboscis extension (PE), and concluded that the first two provide the most consistent measurements of food intake [48••]. A combination of such feeding assays with reverse genetics has been used to characterize the role of TrpA1 in the avoidance of bitter tastants [49,50]. Other assays use video tracking of adult and larval feeding behavior [51,52].

The observations of adults in response to odors can be complemented with electrophysiological recordings of single sensilla in antennae in response to individual volatile chemicals or mixtures thereof. In comparative studies the neuronal response of herbivorous species *S. flava* and *D. suzukii* was higher towards plant-derived odors than to yeast-derived odors, whereas the opposite was true for *D. melanogaster* [11,53].

Conclusion

We have provided an overview of comparative genomic resources and *Drosophila* molecular tools available that could be harnessed for the identification and subsequent verification of genes involved in the evolution of herbivory and diet specialization in insects. When used iteratively, these resources and tools should allow for the systematic characterization of genes involved in all aspects of herbivory (Figure 1).

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