INVITED REVIEW

We can’t all be supermodels: the value of comparative transcriptomics to the study of non-model insects

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Abstract

Insects are the most diverse group of organisms on the planet. Variation in gene expression lies at the heart of this biodiversity and recent advances in sequencing technology have spawned a revolution in researchers’ ability to survey tissue-specific transcriptional complexity across a wide range of insect taxa. Increasingly, studies are using a comparative approach (across species, sexes and life stages) that examines the transcriptional basis of phenotypic diversity within an evolutionary context. In the present review, we summarize much of this research, focusing in particular on three critical aspects of insect biology: morphological development and plasticity; physiological response to the environment; and sexual dimorphism. A common feature that is emerging from these investigations concerns the dynamic nature of transcriptome evolution as indicated by rapid changes in the overall pattern of gene expression, the differential expression of numerous genes with unknown function, and the incorporation of novel, lineage-specific genes into the transcriptional profile.

Keywords: RNA-Seq, non-model organism, differential expression, comparative transcriptomics, NGS, insects.

Introduction

How phenotypic diversity is generated is a major question in modern biology. The basis of such diversity in insects is an interesting question not only evolutionarily, but also in an applied context, because insects are major agricultural pests and vectors of many human diseases. As the genomics revolution has progressed, it has become clear that simply detecting the genetic differences between two phenotypically divergent forms often tells us little about the underlying basis of the divergent trait. This is particularly true in cases where highly similar (or even identical) genomes give rise to different phenotypes, as is seen in the polyphenism of eusocial species and in the radical sexual dimorphism observed in many insect orders. Above the species level, two taxa may exhibit minimal differences at the nucleotide level yet show extreme phenotypic divergence. King & Wilson (1975) first pointed out that the basis of many phenotypic changes might not be harboured in simple point mutations in structural proteins, but rather centred in regulatory gene expression. Recently this phenomenon has been invoked in insect evolution (Catalan et al., 2012; Hardison & Taylor, 2012; Saminadin-Peter et al., 2012; Glaser-Schmitt et al., 2013), and a full understanding of the striking phenotypic evolution observed in insects will require both genomic and transcriptomic approaches.

In the very recent past comparative studies of gene expression were limited to a few organisms, but the advent of next-generation sequencing, combined with continuous progress in RNA sequencing (RNA-Seq) data quantity and quality (Picelli et al., 2013), have democratized the technology. It is now possible for researchers outside the model organism canon to use comparative transcriptomics, and studies of a variety of insect traits have recently benefited from this approach. Because neither a reference genome nor extensive knowledge of the biology of the targeted insect are needed to accomplish a detailed transcriptome analysis, numerous and phylogenetically diverse insect species have been examined in the past decade. By tracking the expression...
of genes across several species or under various conditions and developmental stages, entomologists have gained valuable insight into the evolution and molecular basis of insect development, physiology and behaviour.

Many of the earliest insect RNA-Seq studies involved simple annotation analysis to categorize the genes expressed in a single tissue in one species. As methods of assembly, annotation and expression quantitation have improved, researchers are increasingly using a comparative approach (among species, sexes, tissue types, life stages, etc.) to isolate the transcriptional basis of phenotypic diversity. In the present review, we examine the areas of insect biology employing this approach, with particular focus on studies highlighting the dynamic nature of transcriptome evolution through rapid changes in gene expression and incorporation of new genes into transcriptional profiles. We include all insect species examined to date, with the exception of the ‘super-model’ species Drosophila melanogaster, whose transcriptomics are discussed in detail elsewhere (Graveley et al., 2011; and summary databases at http://flybase.org/static_pages/feature/previous/articles/2010_03/RNA-seq_data.html). Important technical aspects of the RNA-Seq method (Box 1) have been discussed in detail in several reviews (Wang et al., 2009; Martin and Wang 2011; Wolf, 2013; etc.), and there are some pitfalls to the approach that must be considered (Box 2). Overall, however, this approach has provided a broadening of opportunities for research at the level of gene expression in an increasingly diverse group of non-model insects.

**RNA-Seq applied to insect biology**

RNA-Seq (also known as whole-transcriptome shotgun sequencing) technology now permeates numerous fields in evolutionary biology and has been used to address questions concerning differential gene expression, phylogenetic relationships, gene creation and gene family evolution, rates of protein evolution, genotype–phenotype association, chromosome organization and the regulation of development. To quantify the escalation of studies examining insect transcriptomes we conducted a literature search using the terms ‘expressed sequence tags’ (‘EST’), ‘microarray’, ‘RNA-Seq’ and ‘transcriptome’. We excluded marker-based analyses such as phylogenomic and population genomic studies from our survey and instead focused on research that examines changes in transcriptional profiles associated with phenotypic differences. This search revealed that most branches of insect diversity research now use transcriptome analysis (Fig. 1). As shown in Fig. 2A, RNA-Seq technology has quickly supplanted previous genomic approaches (e.g. EST sequencing and microarray cDNA hybridization) as the preferred method to examine gene expression patterns.

The broad utility of RNA-Seq is illustrated by the diversity of insect groups and research questions addressed with this methodology (Fig. 2B). Much of the insect research using RNA-Seq reflects an applied focus on traits of importance to human health or agriculture (e.g. blood meal dynamics, vector-borne infection and resistance to pesticides), but other areas in which RNA-Seq is popular involve a more basic understanding of the evolutionary and behavioural biology of insects (e.g. caste differentiation, gregariousness, olfaction and diapause). We review recent work in these fields by focusing on three critical aspects of insects’ evolutionary success: morphological development and plasticity, physiological response to the environment, and sexual dimorphism.

**Development and plasticity**

*Morphological development*

Even insects with apparently simple hemimetabolous development undergo striking changes as they mature from egg to adult, and recent studies have compared *de novo* assembled transcriptomes across different developmental stages to identify the genes and signalling pathways involved (Chen et al., 2010; Ewen-Campen et al., 2011; Zeng et al., 2013). Most studies to date have involved the Holometabola. Zheng et al. (2012) compared gene expression between larval and pupal stages of the tephritid fruit fly Bactocerca dorsalis and identified a large number of differentially expressed genes, most involved in metabolic pathways (e.g. oxidative phosphorylation and glycolysis). Li et al. (2013) compared the transcriptomes of four developmental stages (embryos, larvae, pupae and adults) of the lepidopteran maize pest Athetis lepidegone and found that most differentially expressed genes were downregulated in larvae and upregulated in pupae. These expression differences were consistent with characteristic features of each life stage; for example, cuticular protein genes were upregulated in larvae and downregulated in pupae. In the only study we are aware of involving hemimetabolous insect development, Bao et al. (2013) conducted sex- and stage-specific transcriptome-wide analysis of immune function genes in the brown planthopper, Nilaparvata lugens, and found that protein families that sense foreign microbial infection (e.g. Peptidoglycan recognition proteins), mediate innate immunity (e.g. clip-domain serine proteases), and immune response effector genes, had relatively high expression levels in nymphs and extremely low levels in eggs. In contrast, some genes contributing to antibacterial defence (e.g. i-type lysozymes) and regulation of immune-response effectors (e.g. Toll genes) had their
Highest expression level in eggs, suggesting that they may have roles in embryogenesis and development as well as in immune function.

Phenotypic plasticity

All insects must complete development to adulthood, but in some species the precise outcome of this developmental trajectory is flexible. Such phenotypic plasticity – the ability of a single genotype to give rise to a range of phenotypes – is an obvious candidate for transcriptomic approaches, because genomics alone cannot explain how various phenotypes are generated. Eusocial insects in the orders Hymenoptera, Isoptera and Hemiptera offer an extreme version of plasticity, known as polyphenism, in...
Polyphenism

Studies of eusociality in Hymenoptera abound (Amdam et al., 2004; Patel et al., 2007; Toth & Robinson, 2007; Boomsma, 2009; Linksvayer & Wade, 2009; Johnson &
In the honey bee, *Apis mellifera*, research on the basis of polyphenism is well advanced, and has been reviewed elsewhere (Evans & Wheeler, 2001; Smith et al., 2008; Simpson et al., 2011). We touch here only on more recent studies using transcriptomic approaches, which have confirmed existing knowledge of the genes involved in eusociality and also identified novel genes and mechanisms. For instance, Simola et al. (2013) conducted genome comparisons of eusocial and solitary species to examine the importance of regulatory vs. compositional changes in the evolution of eusociality. They found nearly 2000 genes (largely involved in neuronal and hormonal functions) with similar regulatory changes in all eusocial vs. solitary insects, and identified specific transcription factors (empty spiracles and cAMP response element-binding protein) that appear to be involved in the evolution of eusociality. In another study, Ferreira et al. (2013) investigated whether expression changes in a conserved toolkit of genes (e.g. vitellogenin, insulin, major royal jelly protein, juvenile hormone (JH), methyltransferases, hexamerin, Malvolio, Amfor and P450) were responsible for caste evolution across social taxa, as suggested by some investigators (Toth & Robinson, 2007; Johnson & Linksvayer, 2010). Using a candidate gene approach, previous studies have concluded that these toolkit genes and processes share conserved roles in caste regulation in many eusocial taxa (Amdam et al., 2004; Toth et al., 2010; Daugherty et al., 2011; Woodard et al., 2011; Huang et al., 2012).

Unbiased surveys of entire transcriptomes of social insects are still rare, however, and experiments using a candidate gene approach are inherently unlikely to find novel explanations for the investigated phenotypes. Thus, some workers have suggested that many of the molecular processes responsible for polyphenism in different social lineages will differ substantially, because extensive molecular and developmental re-wiring may be required in the evolution of caste commitment and eusociality (Boomsma, 2009; Linksvayer & Wade, 2009), and that each lineage may have a unique set of genes responsible for polyphenism. In the case of the primitively eusocial wasp *Polistes canadensis*, Ferreira et al. (2013) identified 2442 genes with caste-biased expression levels. Seventy-five percent of these were novel genes, and there was

![Phylogenetic tree showing the broad phylogenetic distribution of insect transcriptomes. Tree modified after Bugwood (http://wiki.bugwood.org/Main_Page). The number of transcriptomes for each insect order was compiled from the literature. Each order with transcriptomes generated independent of 1KITE (http://www.1kite.org) and surveyed as part of our literature search (key words: ‘transcriptome’, RNA-Seq’, microarray’, or ‘454’; November 2013) has a pie graph associated with it. The green (dark) section of the graph represents the number of species with transcriptomes generated for that order by 1KITE (http://www.1kite.org/downloads/1KITE_species.txt; November 2013). The blue (light) section represents the number of taxa with transcriptional studies done independently of the 1KITE initiative. The number in small font indicates the number of taxa slated for study by the 1KITE initiative. The number in large font represents the number of 1KITE species examined or slated for examination in a given order, where no studies independent of 1KITE have been done.](http://wiki.bugwood.org/Main_Page)
very little overlap in the identity and expression pattern of caste-biased genes from *P. canadensis* and those in other social hymenopterans. Only 6.5% of honey bee caste-biased genes were also caste-biased in *P. canadensis*, and there was no correlation with caste-biased genes of the eusocial fire ant, *Solenopsis invicta*.

Isoptera (termites) have also been the focus of research on phenotypic plasticity with respect to social behaviour (Korb *et al.*, 2009; Husseneder *et al.*, 2012; Tarver *et al.*, 2012; Sen *et al.*, 2013). Termites exhibit JH-dependent caste differentiation, and Tarver *et al.* (2012) examined the role of a termite-specific cytochrome P450 gene (Cyp15F1) in this process. Genes in the cytochrome P450 family 15 are known to play a role in JH biosynthesis and degradation, and elevated JH titres in *Reticulitermes flavipes* workers induce differentiation into the soldier caste via the intermediate presoldier stage. Tarver *et al.* (2012) tested whether expression levels of Cyp15F1 change in response to primer pheromones derived from the heads of soldiers. They found that expression levels of Cyp15F1 were elevated by one soldier pheromone (CAD), and that RNA interference (RNAi) against Cyp15F1 reduced presoldier formation by 78% compared with the no-RNAi control. The authors conclude that primer pheromones, acting as socio-environmental cues, directly influence Cyp15F1 expression and help regulate caste differentiation by mediating JH signalling.

**Phase switching**

Phase change in locusts can be induced rapidly and is fully reversible. The solitary phase, in which locusts actively avoid encountering each other and are cryptically coloured, may persist for many generations. When population densities increase to the point where avoidance is impossible, a shift to the gregarious phase, in which locusts are brightly coloured and can accumulate into devastating swarms, is triggered. While the complete shift from one phase to another – which involves changes in behaviour, colour, morphology, life history and physiology (Uvarov, 1966) – takes several generations, behavioural changes are evident within hours of the phase change-inducing stimulus (Roessingh *et al.*, 1998). Although the number and function of the genes involved in this transition are not yet thoroughly known, two recent studies detected similar numbers of transcripts with phase-related expression differences: Badisco *et al.* (2011) conducted a microarray-based comparison of genes expressed in the central nervous system and found 214 genes (out of 20 755) differentially expressed in solitary and gregarious phases, while Chen *et al.* (2010), using a *de novo* approach, found 242 (out of 11 490) genes with expression differences. Wang *et al.* (2014) examined methylomes (i.e. DNA methylation patterns), brain transcriptomes and alternative premessenger RNA splice transcripts of solitary and gregarious locusts. Many of the genes that displayed differences are involved in the regulation of cytoskeletal microtubules (essential neuronal structures involved in neuronal polarization, development and plasticity). The authors conclude that the abundant differences between solitary and gregarious locusts in expression levels, methylation states and splice forms contribute to the neuronal plasticity that underlies the behavioural changes required for phase switching.

Consistent with the need to respond to the diverse sensory inputs inherent in swarming and migration, genes
involved in environmental interaction are upregulated in gregarious locusts (Chen et al., 2010; Badisco et al., 2011; Guo et al., 2011). Such genes include those associated with the regulation and development of neuronal structures; regulation of neurotransmitter activities; and detection of chemosensory cues. High expression levels in these categories might help gregarious locusts deal with sensory complexity, because enhanced nervous system plasticity should facilitate rapid adaptation to new environments and stimuli. Acute stress genes [HSPs and pathogen resistance proteins (Badisco et al., 2011)] are also upregulated in gregarious locusts, possibly mitigating the increase in disease risk associated with swarm life. In contrast, ‘anti-aging’ genes, including those associated with oxidative stress resistance, detoxification and ana-bolic renewal (Badisco et al., 2011) are downregulated in gregarious locusts, which may represent a trade-off to free metabolic resources for other functions at the cost of a reduction in lifespan (Uvarov, 1966).

Physiological response to the environment
Response to environmental stress and infection
Insects can respond to environmental stress in many ways. Stressors may be predictable (e.g. seasonal changes in climate, bacteria associated with specific environments) or unpredictable (e.g. unseasonable temperatures, viral infection), and some may be constant (e.g. freezing temperatures in polar environments); thus, the particular environment of each species will determine the genes that are differentially expressed in response to stress. Several studies have explored changes in gene regulation in response to cold stress (Timmermans et al., 2009; Teets et al., 2012; Dunning et al., 2013; Teets & Denlinger, 2013). Teets et al. (2012) examined gene expression changes in response to extreme cold in the Antarctic midge, Belgica antarctica, which is the world’s southernmost insect and the only insect known to be endemic to Antarctica. They found upregulation of cellular recycling pathways and coordinated upregulation of numerous genes involved in autophagy (Teets & Denlinger, 2013), suggesting that autophagy and related processes are important aspects of stress tolerance in the Antarctic environment. The authors compared these results with data from the Arctic collembolan, Megaphorura arctica, and found very little overlap in expression profiles, indicating that these polar species have developed different gene expression mechanisms to withstand similar desiccating conditions. High temperatures can be as dangerous as low ones, and recent transcriptome studies have shown that, in spite of their name, antifreeze proteins play a role in tolerance of both high and low temperatures (Ma et al., 2012; Qiu et al., 2013).

In predictable environments, dormancy for part of the year might be a reasonable response to environmental stress. Diapause and quiescence are both forms of dormancy that allow insects to survive stressful conditions, but they are distinct in that diapause involves a pre-programmed developmental arrest that is anticipatory in nature, while quiescence is characterized by an immediate developmental arrest in response to unfavourable conditions. These processes have been examined by comparing the transcriptomes of diapausing and quiescent organisms (Poelchau et al., 2013b), and of developmental stages in different phases of diapause (Poelchau et al., 2013a). For instance, Gong et al. (2013) compared the transcriptomes of the diapausing and non-diapausing orange wheat blossom midge, Sitodiplosis mosellana, an economically important wheat pest. In diapausing individuals, Gong et al. found upregulation of heat shock proteins, carbohydrate metabolism genes, and genes involved in the suppression of JH signalling and response.

The expression of immune response genes (i.e. those transcribed in response to infection by viruses, bacteria or parasites) has been examined in several species (Table S1). In the harlequin ladybird beetle, Harmomia axyridis, a widespread invasive species that has acquired strong resistance to bacterial and fungal infection, Vilcinskas et al. (2013) compared transcriptome profiles before and after infection. Their findings indicate that immunity evolved through the rapid expansion of genes coding for antimicrobial peptides and proteins. Similarly rapid evolution of immune response genes was also found in studies of Nasonia vitripennis. Sackton et al. (2013) found that many novel (i.e. found only N. vitripennis) immune genes were induced by infection, producing a species-specific immune system for this wasp.

Blood meal dynamics
Because blood-feeding mosquitoes serve as vectors for malaria, West Nile Virus, and other diseases, the transcriptional regulation of blood feeding is of particular interest to human health. Two recent studies examined the role of olfaction in blood meal timing in Anopheles gambiae: Rinker et al. (2013) found that moderate changes in odorant receptor (Or) transcript abundance can modulate oviposition behaviours after a blood meal, and Rund et al. (2013) identified well-defined cyclic expression patterns of Ors and odorant binding proteins, a family of soluble proteins found in insect olfactory organs. Given that the peaks in expression correspond with times of increased blood-feeding behaviour, such cyclic expression may be responsible for coordinating the olfactory system with An. gambiae’s pre-dusk/dusk circadian niche. Researchers seeking targets for control measures against Aedes aegypti, the principal mosquito vector of dengue viruses,
have examined fat body transcriptomes in blood-fed and non-blood-fed *Ae. aegypti* (Price et al., 2011), while other studies characterizing blood feeding have examined salivary gland and midgut tissues (Table S1).

**Response to plant defence and pesticides**

Although the ability of herbivores to cope with naturally occurring plant defences and the evolution of resistance to synthetic insecticides might seem like separate topics, recent research using RNA-Seq has revealed a surprising amount of overlap between them (Bass et al., 2013; Dermauw et al., 2013). For example, the spider mite *Tetranychus urticae* is polyphagous, and many strains have evolved resistance to all insecticides. To investigate whether the genes that allow *T. urticae* to feed on many chemically defended hosts are also involved in its ability to rapidly evolve pesticide resistance, Dermauw et al. (2013) compared transcriptomes from two different insecticide-resistant strains to those of a strain selected for five generations on tomato (a challenging host for *T. urticae*). They found >40% of the differentially regulated genes were shared by all three groups, and prominent amongst these were some ‘usual suspects’ commonly implicated in detoxification or transport of xenobiotics (e.g. cytochrome P450s, carboxyl/cholinesterases, and glutathione-S-transferases), although genes of unknown function had some of the most marked expression changes. A more striking result, however, was the detection of expression differences in some previously unexplored gene categories, namely binding proteins (e.g. lipocalins) and transport proteins (e.g. the major facilitator superfamily). While the role of these proteins in polyphagy and insecticide resistance is not yet known, their detection – which would have been impossible using a candidate gene based approach – illustrates the value of unbiased whole transcriptome comparisons.

Based on such results, it seems likely that many of the studies that have looked only at the transcriptional response to either naturally occurring plant compounds or only at the response to insecticides will in fact shed light on the reciprocal question. By characterizing differential gene regulation in pest insects in response to plant defences, new targets for pest management could emerge. Many recent studies have examined gene expression changes associated with feeding on well-defended host plants. Chi et al. (2009) found that feeding on defended soybean is energetically costly for the cowpea bruchid, *Callosobruchus maculatus*: the expression of structural, defence and stress-related genes was much lower on defended plants, suggesting that insects reallocate resources to deal with plant compounds at a cost to their ability to respond to other potential challenges. In the case of the whitefly, *Bemisia tabaci*, however, Alon et al. (2012) found that expression changes in response to phenylpropanoids (a large group of plant secondary defence metabolites) do not appear to be costly. They examined reproductive performance and transcriptome profiles after feeding on phenylpropanoid-enriched tobacco and concluded that the resulting elevated expression of genes involved in metabolism, protein synthesis and defence was not accompanied by reduced reproductive performance.

The response of the fall armyworm, *Spodoptera frugiperda*, to toxins present in maize was examined by Feschemyer et al. (2013). They found upregulation of genes involved in the production of midgut constituents (to counteract the peritrophic membrane depletion the maize toxins cause) and of genes involved in the production of digestive enzymes (to help clear the toxins more rapidly). Similarly, Asian rice gall midges, *Orseolia oryzae*, feeding on resistant vs. susceptible rice varieties upregulate genes for proteolysis and protein phosphorylation, possibly in an attempt to break down the toxic proteins more quickly (Sinha et al., 2012).

Some researchers have used the transcriptional response of herbivores to a novel or recently adopted host as a proxy for the evolution of host plant range, hypothesizing that the genes expressed might encompass those involved in historical transitions to new hosts (Liu et al., 2010; Matzkin, 2012; Whiteman et al., 2012; Celorio-Mancera et al., 2013; Luan et al., 2013). These studies involve the examination of experimentally manipulated insects using specific plants as hosts. For instance, (Matzkin, 2012) looked at host range evolution in the cactophilic fruit fly, *Drosophila mojavensis*, which exhibits population-level specialization on different cactus species. Matzkin found that >20% of the genome was differentially regulated in response to a challenging cactus species. Genes associated with mRNA binding and toxin response were upregulated, while genes associated with energy and carbohydrate metabolism and structural components were downregulated. In another study, Celorio-Mancera et al. (2013) examined the evolution of host range in the butterfly *Polygonia c-album*, a polyphagous, widely distributed butterfly. They compared the transcriptomes of larvae feeding on an established host plant (*Urtica dioica*) vs. one that represents a recent evolutionary shift onto a very divergent host (*Ribes uva-crispa*). Like Dermauw et al. (2013) they found unexpectedly high levels of differential regulation of previously unsuspected proteins, namely genes coding for transporter and membrane-binding proteins (including major facilitator superfamily and monocarboxylate types), in response to the newer host. In addition, they found upregulation of cuticular proteins, which they suggest may strengthen the insect gut and prevent toxic plant compounds from entering the insect body.

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Sexual dimorphism

Sexual selection is the driving force responsible for a vast array of novel morphological and behavioural phenotypes found throughout insects (Wilkinson & Dodson, 1997; Emlen, 2008; Allen et al., 2011). The extent of sexual dimorphism ranges from minor differences in somatic structures to dramatic showy or exaggerated characters – such as beetle horns, butterfly wing patterning and cricket calls – involved in competition, sexual advertisement and mate selection. Despite the widespread study of sexual dimorphism, we still know little about how variation at the molecular level controls this morphological diversity. A fundamental aspect of the genetic basis of sexual differences is that males and females have virtually identical genomes. Other than the few genes located on the sex-limited copy of the heteromorphic sex chromosomes (e.g. the Y chromosome in XY sex determination systems), there are no genes that exist exclusively in only one sex. As a result, sexual dimorphism is shaped primarily by differential patterns of gene expression. Research examining the specifics of sexual differences in gene expression has flourished recently (for reviews, see Ellegren & Parsch, 2007; Parsch & Ellegren, 2013), motivated by the advent of next-generation genomic techniques and an interest in a number of different evolutionary phenomena, including: the resolution of sexually antagonistic selection pressures; the molecular evolution of reproductive proteins; the chromosomal distribution of sex-biased genes; and the origin and evolution of dosage compensation. The vast majority of this research has focused on Drosophila, but RNA-Seq offers the opportunity to explore these topics in other insect systems distinguished by dramatic sexually dimorphic phenotypes. Below we summarize the details associated with much of this research and highlight some recent studies on non-Drosophila systems.

Genetic basis of sexual dimorphism

Sexually dimorphic traits arise because different selective pressures affect male and female phenotypes (Lande, 1980). Differences in gene expression between the sexes (termed sex-biased or sex-specific gene expression) evolve to resolve the conflict between male- and female-specific adaptive peaks so that each sex can approach its own adaptive optima (Parsch & Ellegren, 2013); therefore, cataloguing genes that are over-expressed in one sex relative to the other in sexually dimorphic tissue can help identify candidate genes and pathways responsible for sexually dimorphic development. Studies in Drosophila have shown that sex-biased gene expression is pervasive and evolutionarily dynamic (Meiklejohn et al., 2003; Ranz et al., 2003; Zhang et al., 2007; Assis et al., 2012). Depending on the study, up to 50% of the genes examined exhibit differential expression between males and females. Substantial differences in the patterns of sex-biased expression are also found between closely related species, particularly for genes serving male reproductive functions (Meiklejohn et al., 2003; Ranz et al., 2003; Zhang et al., 2007; Assis et al., 2012). These studies, however, have focused primarily on whole-body gene expression where much of the sex-biased expression results from reproductive tissues. Few studies, even in Drosophila, have examined sex-biased expression in specific somatic tissues (but see Chang et al., 2011), and fewer still in a somatic tissue with respect to a specific sexually dimorphic phenotype in an attempt to associate expression differences with phenotypic variation.

In non-Drosophila insects, we are aware of only two studies comparing male and female expression levels at a genomic scale in specific (not whole-body) somatic tissue. Baker et al. (2011) examined sex-biased gene expression in several tissues of the mosquito, An. gambiae, and found that, depending on the tissue, 5–15% of the genome was differentially expressed between males and females. A more directed approach was taken by Wilkinson et al. (2013) in an attempt to understand the expression differences underlying extreme sexual dimorphism in the eyestalks of stalk-eyed flies (Diopsidae). Using microarrays designed from an EST library (Baker et al., 2009), they identified nearly 900 genes that were sex-biased in the developing eyestalk tissue but not in the adult head. As might be expected because males possess a larger eyestalk, male-biased genes were over-represented for genes involved in growth and mitochondrial function. Results from both of these studies suggest that transcriptome analysis provides a valuable tool for understanding the overall architecture of sexual dimorphism, but, because sex-biased expression is so widespread, such analyses are unlikely to directly identify candidate genes. It is also important to note that the causal loci directly responsible for sexually dimorphism may not exhibit any expression differences between the sexes, but rather exert their influence through cis-regulatory effects or alternative splicing of pre-mRNA. It will be critical for future studies to identify essential upstream regulators of sexual dimorphism and exaggerated traits, such as doublesex and Insulin-like receptor (Emlen et al., 2012; Gotoh et al., 2014), and to examine the transcripational impact of these genes through functional manipulation (e.g. RNAi).

Evolution of reproductive proteins

Genes involved in mating and reproduction, particularly for males, are among the most rapidly evolving loci in the genome, and often show evidence of positive selection (Haerty et al., 2007; Singh & Artieri, 2010). Because of
their adaptive significance, reproductive proteins have been the subject of many evolutionary studies, and transcriptome analyses offer an efficient way of examining their molecular evolution. While few non-<i>Drosophila</i> insect studies have directly measured sex-biased gene expression on a genomic scale, examination of transcript diversity in a sexually dimorphic tissue for one sex is much more common. These studies include gene expression analysis of the sex pheromone glands in the moth <i>Heliothis virescens</i> (Vogel et al., 2010), the developing horn tissue in the beetle <i>Onthophagus taurus</i> (Choi et al., 2010), and the male reproductive organs of several insects — two <i>Heliconius</i> species (Walters & Harrison, 2010, 2011), <i>Ceratitis capitata</i> (Scolari et al., 2012), <i>Lutzomyia longipalis</i> (Azevedo et al., 2012), <i>Teleopsis dalmanni</i> (Baker et al., 2012; Reinhardt et al., 2014), <i>Teleogryllus oceanicus</i> (Bailey et al., 2013) and two <i>Gryllus</i> species (Andres et al., 2013). Protein sequences identified through transcriptome surveys can be compared with protein sequences from other species to test various hypotheses of molecular evolution. For instance, Walters and Harrison (2011) recently used a transcriptome analysis of seminal fluid proteins in <i>Heliconius</i> butterflies to examine the correlation between mating system and the rate of protein evolution. Contrary to expectation, they found that rates of protein evolution were not accelerated in species with promiscuous vs. monogamous female mating behaviour.

Another critical feature of transcriptome studies is that they facilitate the identification of lineage-specific genes that have no direct homologue in other insect groups. Novel genes can arise in many ways (e.g. gene duplication, lateral gene transfer, de novo gene creation from noncoding regions of DNA) and may rapidly acquire adaptive functional roles (Ranz & Parsch, 2012; Chen et al., 2013). Studies in <i>Drosophila</i> have shown that novel genes represent a substantial component of male-biased gene expression and play a critical role in spermatogenesis. A recent study in stalk-eyed flies (Baker et al., 2012) highlights the ability of RNA-Seq to uncover novel genetic diversity associated with reproductive proteins. More recently, testes transcriptomes from additional stalk-eyed fly species have been analysed, and numerous lineage-specific novel genes have been found (see Fig. 3 for an example). The gene family represented in Fig. 3 comprises homologues of the <i>D. melanogaster</i> gene, <i>Nucleosome assembly protein 1</i> (Nap1), an important histone chaperone gene that is expressed in the testes (Kimura, 2013). In <i>Drosophila</i> and the mosquito species, <i>Nap1</i> is present as a single homologue, while stalk-eyed fly species exhibit as many as nine paralogues of <i>Nap1</i>. Overall, the tree suggests there have been at least 14 duplications of the <i>Nap1</i> gene within the family, and it appears these new genes function primarily in spermatogenesis. Of the seven paralogous copies in <i>T. dalmanni</i>, the one with the closest homology to the <i>D. melanogaster</i>
Nap1 protein is expressed ubiquitously throughout the body, while the other six copies exhibit testes-specific gene expression (Fig. 3). DNA undergoes dramatic condensation during spermatogenesis involving the replacement of histones with protamines (White-Cooper, 2010), so it is possible these new Nap1 proteins play some role in this process. Overall, the use of transcriptomes for gene discovery has some inherent limitations because variation in expression levels across taxa will impact their representation in any gene family analysis. Even with this caveat, transcriptome surveys are an incredibly powerful tool for exploring the genetic diversity that is unique, and therefore of particular relevance, to non-model organism groups.

**Evolution of sex chromosomes**

Perhaps the most widespread use of sex transcriptome analysis involves addressing evolutionary questions regarding the origin and composition of sex chromosomes. Most insect species have a genetic sex determination system with one sex possessing heteromorphic sex chromosomes comprised of one gene-rich chromosome and one degenerate, heterochromatic, gene-poor chromosome (for reviews of the evolutionary processes driving this state see: Charlesworth, 1991; Charlesworth et al., 2005; Bachtrog, 2006). Which sex carries the heteromorphic pair varies among taxa (e.g. XY males/XX females in Diptera, ZZ males/ZW females in Lepidoptera), but in either case, the result is a dose imbalance for sex-limited genes in the heterogametic sex. To offset the potential fitness costs of having a single gene copy, dosage compensation mechanisms that balance gene expression levels across the genome are expected to evolve.

The abundant variation found among insects in both sex-determination systems and sex chromosome composition (Baker & Wilkinson, 2010; Gemppe & Beye, 2011; Sahara et al., 2012; Vicoso & Bachtrog, 2013) has motivated a number of studies using RNA-Seq to examine the evolution of dosage compensation by comparing the expression levels of autosomal and sex-linked genes (Vicoso & Charlesworth, 2009; Prince et al., 2010; Harrison et al., 2012; Wilkinson et al., 2013). These studies have confirmed the observation that hypertranscription of the heterogametic sex chromosome occurs in XY systems (Prince et al., 2010; Wilkinson et al., 2013), but not ZW systems (Zha et al., 2009; Harrison et al., 2012). However, the mechanism of dosage compensation appears to differ between insect groups: beetles exhibit hypertranscription of the X chromosome in both sexes (Prince et al., 2010), while, in stalk-eyed flies, hypertranscription of the X chromosome appears to occur only in males (Wilkinson et al., 2013). In addition, ambiguity remains regarding the absence of dosage compensation in ZW species (Walters & Hardcastle, 2011). Transcriptome analyses in several more insect groups will be needed to map the evolutionary origin of dosage compensation systems and to understand the specific genetic mechanisms controlling dosage effects.

The existence of heteromorphic chromosomes affects the evolution of sex-biased gene expression well beyond the effects of dosage compensation. Sex-linked genes in species with heteromorphic chromosomes are found more often in the homogametic sex (e.g. the X chromosome spends 2/3 of its time in females in XY systems), a condition that has strong implications for the resolution of sexually antagonistic selective pressures (Rice, 1992; Bonduriansky & Chenoweth, 2009). Sex chromosomes are predicted to be a preferred location for sex-biased genes, and early transcriptome surveys of sex-biased gene expression in *Drosophila* revealed dramatic differences in the distribution of sex-biased genes, with the X chromosome significantly over-represented for female-biased genes (Paris et al., 2003; Ranz et al., 2003; Sturgill et al., 2007). This research sparked an interest in the composition of sex chromosomes, and every study to date has found some form of differential representation of sex-biased genes on the sex chromosomes. For XY species, there is evidence of over-representation of female-biased genes on the X chromosome (Prince et al., 2010; Wilkinson et al., 2013) and under-representation of male-biased genes on the X chromosome (Magnusson et al., 2012). In contrast, the Z chromosome in the silk moth, *Bombyx mori*, is enriched for genes that function in the testes and is the source of excessive gene movement of female-biased genes onto autosomes (Arunkumar et al., 2009; Wang et al., 2012), while pea aphids (*Acyrthosiphon pisum*), which have an XO sex-determination system, have a highly masculinized X chromosome that is consistent with predictions based on the unusual mode of X chromosome inheritance in this species (Jaquiery et al., 2013). Given the rapid turnover in sex chromosome identity within insects (Kaiser & Bachtrog, 2010; Pease & Hahn, 2012; Vicoso & Bachtrog, 2013), the effect of chromosomal location on the pattern of sex-biased gene expression is likely to provide a major evolutionary force shaping the phenotypic differences between the sexes.

**Conclusion**

The democratization of RNA-Seq technology in terms of affordability, accessibility and interpretability, has paved the way to exciting progress in understanding the transcriptional basis of phenotypic diversity in non-model insects. Over the past few years, the number of published studies using this approach has more than doubled, allow-
ing researchers to address previously intractable questions. RNA-Seq has been especially useful in cases where extreme phenotypic divergence occurs between individuals with similar or identical genomes, such as sexual dimorphism and social polyphenism.

RNA-Seq has been used in a variety of ways in non-model insects. At its most fundamental level, RNA-Seq allows for the identification, annotation and comparison of the sets of genes expressed in different species, individuals or phenotypes. Such annotation-based studies have led to the identification of gene families that are evolving especially rapidly, and thus are plausible candidates for explaining the evolution of divergent phenotypes. Beyond cataloging the genes present in a transcriptome, RNA-Seq has further been used to compare the expression of genes across species, under various environmental conditions, and at different developmental stages. A third, and very recent, application of RNA-Seq is for examination of alternative pre-messenger RNA splicing. Alternate splice sites give rise to different protein isoforms, and these can radically alter protein function. In humans, recent studies suggest that >90% of all genes encode two or more proteins via alternative splicing of pre-messenger RNA (Wang et al., 2008), and in cases where both genetic sequence and gene expression levels are identical, it may be the case that alternative splicing is responsible for phenotypic divergence. While detailed surveys of the prevalence and impact of alternative splicing on insect phenotypes are still lacking, work on the Dscam gene, which plays a role in axonal guidance and can generate 38 016 distinct mRNA isoforms in D. melanogaster (Schmucker et al., 2000), suggests that hypervariable splicing of this gene is common throughout the Arthropoda (Armitage et al., 2012). In non-model insects, preliminary results from the tarnished plant bug (Lygus lineolaris) suggest that alternative splicing may contribute to the divergence of odorant binding proteins (Hull et al., 2014). Because odorant binding proteins mediate odorant detection and discrimination, alternative splicing of the genes coding for these proteins could affect oviposition, host plant use, mate preference and many other evolutionarily important behaviours.

One of the most striking results of the increasingly large number of de novo (rather than candidate-based) RNA-Seq studies published to date is the realization that many genes of unknown function are being expressed in virtually every species that has been examined. Because transcriptome annotation necessarily relies on sequence similarity to previously identified genes, the finding that 50% (or more) of the differentially expressed (or alternatively spliced) genes in one’s study are unannotated means that it is difficult to know what role, if any, these genes have in explaining the phenotype of interest. Now that the most vexing aspects of RNA isolation, library preparation, sequencing, assembly and analysis are fairly well worked out, the next great challenge for those who study non-model organisms is to simplify the process of figuring out just what all those genes are for.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.