Something old, something new, something borrowed, something red: the origin of ecologically-relevant novelties in Hemiptera

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Abstract

Comparative transcriptomics, applied in an evolutionary context, has transformed the possibilities for studying phenotypic evolution in non-model taxa. We review recent discoveries about the development of novel, ecologically-relevant phenotypes in hemipteran insects. These discoveries highlight the diverse genomic substrates of novelty: 'something old', when novelty results from changes in the regulation of existing genes or gene duplication; 'something new', wherein lineage-restricted genes contribute to the evolution of new phenotypes; and 'something borrowed', showcasing contributions of horizontal gene transfer to the evolution of novelty, including carotenoid synthesis (resulting in 'something red'). These findings show the power and flexibility of comparative transcriptomic approaches for expanding beyond the 'toolkit' model for the evolution of development. We conclude by raising questions about the relationship between new genes and new traits and outlining a research framework for answering them in Hemiptera.

Introduction: Setting the evolutionary and developmental stage

Hemiptera is a fantastically diverse insect clade, with more than 100,000 described species (Fig. 1). The original motivation for adding a hemipteran to the evo-devo pantheon was their close relationship to holometabolous insects, allowing inferences about major evolutionary transitions, such as the origin of complete metamorphosis. More recently, researchers have begun to analyze the origin of hemipteran-specific traits, including many traits that contributed to their ecological success. One key innovation underlying their radiation is piercing-sucking mouthparts. These enabled diversification across feeding niches, which are closely integrated with the emergence of additional novel phenotypes (*e.g.*, defense mechanisms, polyphenisms,

and mutualisms) [1,2].

In this review, we highlight recent progress in identifying the developmental basis for novel traits of ecological and evolutionary significance across Hemiptera. We begin with a brief overview of the shift from candidate genes to comparative transcriptomics as a starting point for research, which has enabled rapid progress on understanding the origin of a wide range of traits. These discoveries not only confirm the varied ways in which 'old' genes are reused, but also show the importance of new genes and genes acquired through horizontal gene transfer in the evolution of novelty. In Hemiptera, the supply of these latter two genomic substrates for novelty is likely enhanced by extensive biotic interactions linked to their feeding habits and dynamic genomes. In the concluding section, we expand our focus from questions about the origin of particular traits to testing broader hypotheses about the relationship between new genes and the evolution and development of phenotypic novelty.

From Candidate Gene Approaches to Comparative Transcriptomics:

A foundational discovery in evo-devo was the existence of an evolutionarily ancient, highly conserved developmental toolkit. This led to an early, productive research paradigm based on candidate genes, which identified myriad instances in which 'old' genes deployed in new contexts can lead to novelty [3]. For example, Hox genes are natural candidates for regulation of segment-specific phenotypes. In hemipterans, loss of a highly conserved Hox gene expression domain underlies evolution of their unique fluid-feeding mouthparts (Fig. 1j) [4] while the appearance of novel Hox gene expression domains is an early step in bacteriocyte development (Fig. 1h,i) [5,6]. These specialized abdominal cells are another hemipteran key innovation, housing endosymbionts essential for sap-feeding bugs. Interestingly, regulation of bacteriocyte

development by Hox genes is inferred to have followed the same complex history as bacteriocytes (Fig. 1a), with origin, loss and reevolution tracing shifts from plant-feeding to predation and back to plant-feeding [6].

More recently, transcriptomic comparisons have provided a way to survey the full repertoire of gene expression to discover the sources of novelty without *a priori* assumptions about the developmental genetic basis for change [7–9]. The general approach is adaptable to a wide range of novel traits, including the developmental basis of phenotypic plasticity [9–11]. Because the approach requires little taxon-specific customization, it is also widely applicable across taxa. In Box 1, we distill this research approach into four key steps (comparative transcriptomics, filtering of differentially expressed genes, evolutionary validation, and developmental validation). A recurrent theme, illustrated by recent studies, is the increased power provided by using multiple transcriptomic comparisons. This research approach provides a flexible, powerful way to rapidly identify loci contributing to the extensive morphological, ecological and physiological novelty within Hemiptera.

Novelty from Old Genes:

Co-option, the deployment of an ancestral gene or gene regulatory network in a new developmental context, is one way in which 'old' genes can produce novel phenotypes. Two recent studies showcase the use of transcriptomic comparisons to test hypotheses about the role of large-scale cooption in generating phenotypic novelty. Large globular cells (LGC) of gall-forming social aphids are a novel defensive cell type with an unusual combination of properties [12] (**). When a breach in the gall occurs, aphid soldiers 'explosively' discharge bodily fluid containing LGCs that burst, then spread the fluid over the breach. Melanization of the resulting

lipid ‘clot’ reseals the gall. Comparisons across multiple cell types support the hypothesis that LGCs have evolved from hemocytes (an ancestral immune cell type) via upregulation of the wound-healing (melanization) pathway and cooption of lipid synthesis pathways that were expressed ancestrally in fat body cells. This colony-level defensive trait thus results from externalization of an individual-level immune system process [12] (**).

The charismatic hemipteran lineage Membracidae (treehoppers) is best known for its striking array of ‘helmets’, a novel three-dimensional projection of the pronotal body wall that functions in defense (Fig. 1m,n). Transcriptomic comparisons across body regions in two taxa supported the hypothesis that the helmet evolved via cooption of wing-patterning genes [8] (**). In the treehoppers, gene expression in the pronotal helmet was divergent from that in its serial homologue, the mesonotum, but similar to gene expression in wings. In a leafhopper, a helmet-less relative of treehoppers, pronotal gene expression most closely resembled mesonotal expression. [8] (**).

A novel trait that has arisen repeatedly across insects is wing polyphenism, reflecting a trade-off between investment in dispersal (long-winged form) and reproduction (short-winged/wingless form). In aphids, transcriptomic comparisons led to identification of a single microRNA that acted as a developmental switch regulating offspring wing phenotype in response to maternal crowding [10] (*). Crowding lowered expression of this evolutionarily highly conserved miRNA, miR-9b, which targets a transporter gene mRNA. The resulting increase in transporter gene expression in fat bodies shifted development to the long-winged form via increased insulin signaling (Fig. 2), an ancestral, environmentally-responsive metazoan signaling pathway [10] (*). Two other links between environmental determinants of wing phenotype and insulin signaling have been discovered in the planthopper *Nilaparvata lugens* [13,14] (Fig. 2), a

hemipteran with an independently evolved wing polyphenism. These studies give mechanistic insight into the evolutionarily recurrent role of the insulin signaling pathway as an integrator of environmental signals regulating polyphenisms in hemipterans [15–18] and insects more broadly [19].

Gene duplication provides another common route to the origin of novel traits from 'old' genes. In Hemiptera, which show a high rate of gene duplication [20–22], a recurring theme is the role of duplication followed by neofunctionalization in the origin of new salivary enzymes. These enzymes have enabled ecological host switching, host range expansion and acquisition of new feeding strategies such as blood-feeding [23,24]. For example, neofunctionalization of proteases has led to venoms and digestive secretions in predatory bugs [25]. Other examples of strikingly enlarged gene families include genes encoding defensive cysteine-rich peptides that have undergone independent expansion in aphids [26] and assassin bugs [27], and genes encoding stylet cuticular proteins [28,29]. In the peach aphid *Myzus persicae*, the gene family encoding RR-2 hard cuticular proteins is greatly expanded and environmentally regulated. On switching to a new host, changes in expression of RR-2 genes occur rapidly at the plant fluid-insect interface, in an aphid-specific organ at the tip of the maxillary stylets. This plasticity contributes to the host-switching success of this rare true generalist sap feeder [30] (**).

Novelty from New Genes:

Gene duplication helps bridge the divide between 'old' and 'new' genes. New genes may originate by relaxed selection and accelerated evolution or the gain or loss of protein domains that is enabled by gene duplication [31]. New genes may also be derived *de novo* from non-coding sequence [32]. The increasingly dense sampling of hemipteran genomes reveals that they

are dynamic, and incorporate a substantial proportion of genes that lack detectable orthology outside the species or lineage [21,33–35]. A broad comparative analysis found that 'new', taxon-restricted, genes are more prevalent in hemipterans than in most other insects [36].

Comparative transcriptomic approaches have led to an increasing appreciation for the role of new genes in the origin of hemipteran novelties. In an elegant study, Santos et al. [7] (**) identified a paralogous pair of taxon-restricted genes required for the development of a novel leg fan in water striders in the genus *Rhagovelia* (Fig. 1k,l). This fan enabled locomotion on the surface of fast-flowing streams. Loss-of-function analyses showed that two genes (*mother of geisha*, a likely hemipteran-specific gene, and *geisha*, its divergent *Rhagovelia*-restricted descendant) are essential for fan initiation and that they have a spatially-restricted expression domain where the fan develops. Evolutionary comparisons confirmed that relatives of these genes were not similarly expressed in closely related species lacking a fan [7] (**). Chen et al. [11] (*) offer an interesting physiological counterpart, also suggesting the importance of new genes for evolution into new adaptive zones. They discovered a family of long non-coding RNAs that lacked detectable orthologues outside of aphids (named Ya genes). This gene family formed a co-expression module that was differentially expressed across host plant species in the generalist peach aphid. Functional analyses showed that aphids inject a subset of the Ya transcripts into plants, where they are transported systemically, ultimately having an effect on the plant that increases aphid fitness [11] (*).

Novelty from Borrowed Genes (including something red):

Genes that are 'new' for a focal lineage may also be borrowed via horizontal gene transfer (HGT), the asexual acquisition of genetic material from a distantly related lineage. A

biologically intimate relationship between the donor and recipient appears to be a key facilitator of HGT [37]. HGT may be more prevalent and thus more likely to contribute to novelty in hemipterans than in many other arthropod taxa because of their large number of biologically intimate relationships (Fig 1b-e), including with endosymbionts, host plants, and fungi and viruses associated with those hosts.

Bacteria and viruses provide a significant source of novel genes that have conferred novel traits on bugs. Often, these "borrowed" genes continue to perform their ancestral function. Examples include the acquisition of biotin synthesis genes in whiteflies [38], peptidases in the blood feeder *Rhodnius prolixus* [23], and some genes for amino acid and peptidoglycan biosynthesis in mealybugs which continue to function in concert with other bacterial genes present in their endosymbionts [39,40]. More remarkable is the acquisition by aphids of a prokaryotic gene encoding a eukaryotic cell toxin, which aphids deploy as an effective defense against parasitoids [41] (*).

A recently discovered example of HGT from a virus to an aphid provides a fascinating instance of an evolutionary transfer of a developmental regulatory function accompanying HGT. Using transcriptomic comparisons between aphid genotypes that differed in their propensity to develop wings in response to maternal crowding, five genes were identified that were upregulated in the most inducible genotypes but not in those that were the least inducible [9] (**). Two of these genes (named *Apns-1* and *Apns-2*) proved to be of viral origin, but were incorporated into the aphid genome [9] (**). Remarkably, the closest known viral relatives of these genes also regulate aphid wing development, but under viral control rather than in response to environmental conditions sensed by the aphids [42]. How these genes interact with other components of the developmental switch regulating the wing polyphenism and how their

187 expression is regulated within the aphid genome are not yet known.

188 Although much rarer, HGT from eukaryotes has also led to hemipteran novelties. Aphids
189 and their close relatives are one of only a handful of metazoan taxa that synthesize carotenoids.
190 This ability resulted from HGT of multiple carotenoid biosynthesis genes from a fungus to an
191 ancestor of aphids and phylloxerids [21,43,44]. One phenotypic manifestation is a conserved
192 red/green color polymorphism (Fig. 1f,g) [43] and carotenoid expression affects aphid fitness
193 [45]. Another example involves a gene encoding a plant cell wall-degrading enzyme
194 (polygalacturonase) that was transferred from fungi to the bug family Miridae [46]. The
195 duplicated descendants of this gene are highly expressed in the salivary glands, increasing
196 nutrient acquisition in this lineage that reverted to plant-feeding from a predaceous ancestor [47].
197 Recent evidence also demonstrates HGT from plants to an ancestor whiteflies of a highly
198 expressed gene that inactivates 28S rRNA [48].

199

200 **Conclusion and future prospects:**

201 True bugs (Hemiptera) are characterized by extensive morphological and ecological
202 diversity, repeated independent origins of many traits, including adaptive phenotypic plasticity,
203 and molecular communication with a diverse array of intimate associates. As many of the
204 examples highlighted above demonstrate, transcriptomic approaches combined with evolutionary
205 and developmental validation provide a way to identify candidate genes and pathways
206 underlying ecologically important novelties, even without prior knowledge about development in
207 the system (Box 1). This approach has led to the discovery that both new genes and 'borrowed'
208 genes are frequently involved in the evolution of new ecologically important traits, while also
209 confirming the role of 'old' genes initially demonstrated through a candidate-gene approach.

These discoveries set the stage for additional work to fully link developmental changes to phenotypic novelty in systems in which ecological consequences of the phenotype are often accessible for study [7,11,30].

To date, the contributions of new and borrowed genes to novelty have been discovered serendipitously through research seeking to understand the origin of specific hemipteran traits. The repeated association of novel genes and novel phenotypes observed in hemipterans [7,11] and other taxa [49,50] stimulates questions about whether facets of this relationship are predictable. Evidence that new genes originated at higher rates in lineages undergoing more radical morphological transformations [51] also motivate the hypothesis of a non-random association between new genes and new traits. Are new genes more likely than evolutionarily older (or younger) genes to regulate development of traits that arose along the same branch of the evolutionary tree? Do the developmental functions of new genes differ in new versus old traits? Do the developmental functions of new versus old genes differ in consistent ways? Are new genes more likely to become integrated at particular points in developmental networks, as suggested by different degrees of transcriptomic conservatism through time [52,53]? Systematic testing of these hypotheses about the developmental and evolutionary consequences of novel genes can be done by comparing the expression or function of new and 'control' genes in a comparative context (Fig. 3). Because of their high frequency of taxonomically-restricted genes [21,33,35,36], hemipterans provide a model clade for such systematic tests.

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Box 1: Experimental Approaches

Transcriptomic comparisons, combined with phylogenetic information and functional analyses, provide a way to rapidly identify interesting candidate loci underlying the extensive morphological, ecological and physiological novelty within Hemiptera. Generalizing from recent studies, we distill this approach into four key steps. For each step, we provide an overview of methodological considerations and examples of successful applications.

Step 1—Comparative RNAseq (panel a). Characterize gene expression in forms with and without the focal trait at relevant developmental stages; if possible, incorporate multiple comparisons. Informative comparisons may come from serial homologues [7,8], direct homologues [8], relatives raised in different environments [9,11,30], individuals representing extremes of variation within a population [9], and non-homologous body regions that share some features with the focal trait [8,12].

Step 2—Candidate gene identification (panel b). Identify genes that are differentially expressed (DE) and filter to identify likely candidates. A common challenge is that the number of DE genes can be quite large. Multiple comparisons (e.g., between both serial and direct homologues) can greatly narrow the candidate set [7,8,16]; other approaches to filtering include expression screening [7], identification of coregulated gene modules associated with the novel trait [11], and testing bioinformatically for overrepresented pathways and functions [8,16].

Step 3—Evolutionary validation (panel c). Map the developmental change on a phylogenetic tree by characterizing candidate gene expression (or presence) in select taxa. The developmental change and the novel trait should occur on the same branch of the phylogenetic tree. The strongest support requires analysis of multiple ingroup taxa, representatives of the closest outgroup taxon, and a more distant outgroup [7].

Step 4—Developmental validation (panel d). Use functional manipulations to test role of the candidate gene or pathway in development of the novel trait. Here, RNAi (reviewed in [54,55] and CRISPR/Cas-9 genome editing have been transformative, because they work with minimal modifications across diverse hemipteran taxa. Strategies to reduce non-specific dsRNA degradation may be necessary to enhance RNAi success in aphids and planthoppers [56–59]. CRISPR/Cas-9 success rates shown for injected individuals (G0) and next generation (G1) [60–62]. Photo of *O. fasciatus* by David Hill (used under CC BY 2.0 license). Step 4 RNAi panel created with Biorender.com.

Figure 1: Hemipteran diversity and novelty

(a) Phylogenetic tree (following [1]) highlighting major groups of hemipterans and several morphological novelties discussed in the text. (b-g) Examples of ecological interactions shaping evolution in Hemiptera, including (b) sap-feeding aphids; (c) mutualism between woolly alder aphid (*Prociphilus tessellatus*) and ants; (d) plant galls induced by elm sack gall aphids (*Tetraneura ulmi*) and (e) grape phylloxerids (*Daktulosphaira vitifoliae*); (f, g) pea aphid (*Acyrthosiphon pisum*) color polymorphism resulting from horizontal gene transfer of carotenoid genes from a fungus. (h-n) Novel traits discussed in the review; (h) early stage pea aphid embryo in mother's ovariole shows developing bacteriocytes (white arrow) expressing Ubx/abd-A (blue) prior to germ band extension; red arrow indicates endosymbionts; green is phalloidin; (i) bacteriocytes (bc, bug nuclei stained blue) harboring endosymbionts (stained green) in the seed bug *Nysius*; (j) scanning electron micrograph of piercing-sucking mouthparts of a lace bug (*Stephanitis nashi*), with grooved labium (Lb) supporting the mandibular and maxillary stylets (Sf); Lm-labrum; (k) increased leg lengths enable water striders to locomote on the water surface; (l) one lineage, *Rhagovelia*, has evolved novel fans on the middle legs that allow surface

locomotion in fast-flowing currents; (m, n) treehopper helmets are a novel three-dimensional outgrowth that may function in defense and that take on a wide array of shapes as shown by (m) *Cladonota machinula* and (n) *Entylia carinata*. Image credits and bug IDs: (a) insect silhouettes from PhyloPic.org; heteropteran copyright Dave Angelini (CC-BY-3); (b) Paul Eisenberg (CC BY 2.0); (c-e,m) Judy Gallagher (CC BY 2.0); (f) Shipher Wu; (g) N. Gerardo; (h) PLoS Open-Access license; from [5]; (i) from [63], permission pending; (j) [64] by permission; (k) David Hill (CC BY 2.0); (l) A. Khila, permission request pending; (f,g,n): public domain.

Figure 2: Environmental effects on insulin signaling regulate wing polyphenisms.

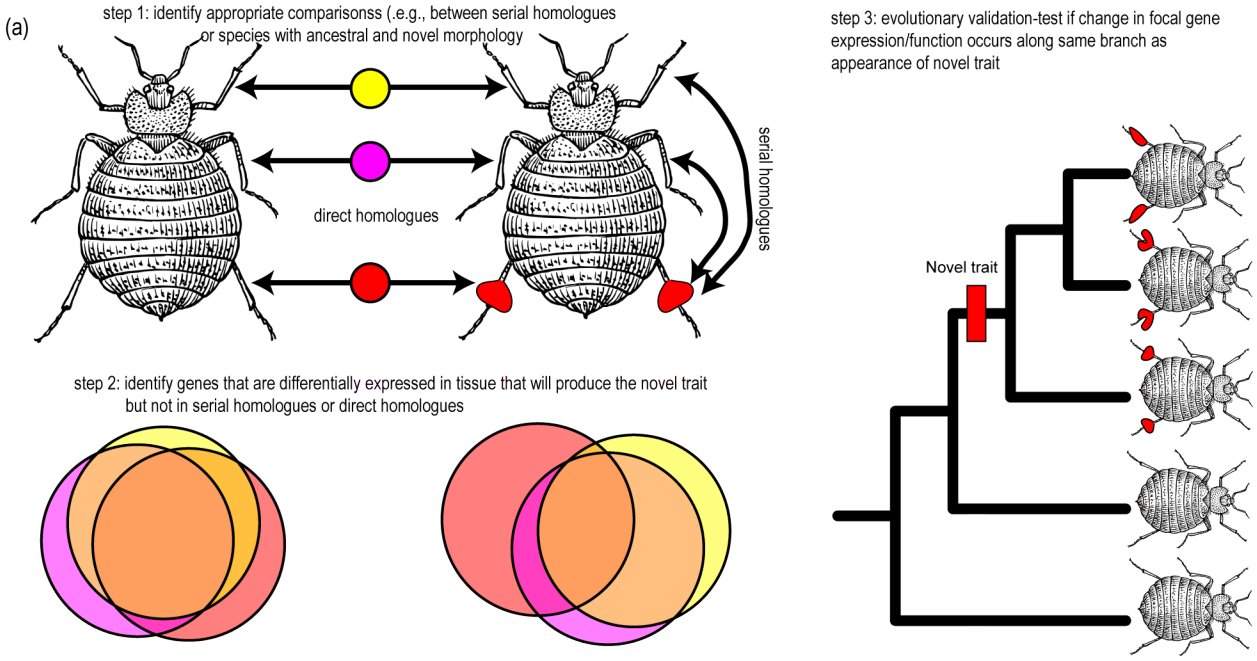
The insulin signaling pathway plays a role in environmentally-cued switching between wing forms, regulating the propensity to develop as a long-winged or short-winged/wingless individual depending on environmental conditions. Recent studies have identified how different environmental triggers interface with insulin signaling at different points in the signaling pathway, with crowding [10] (*) and low diet quality [13] switching development to the dispersive form and wounding switching development to the short-winged form [14]. Colors indicate which wing form (blue-long; red-short) is promoted by the environmental condition or gene activity in the brown planthopper, *Nilaparvata lugens* [18]. *N. lugens* images from Bugwood.org, used with permission.

Figure 3: Comparative approach for testing hypotheses about the developmental role of new genes

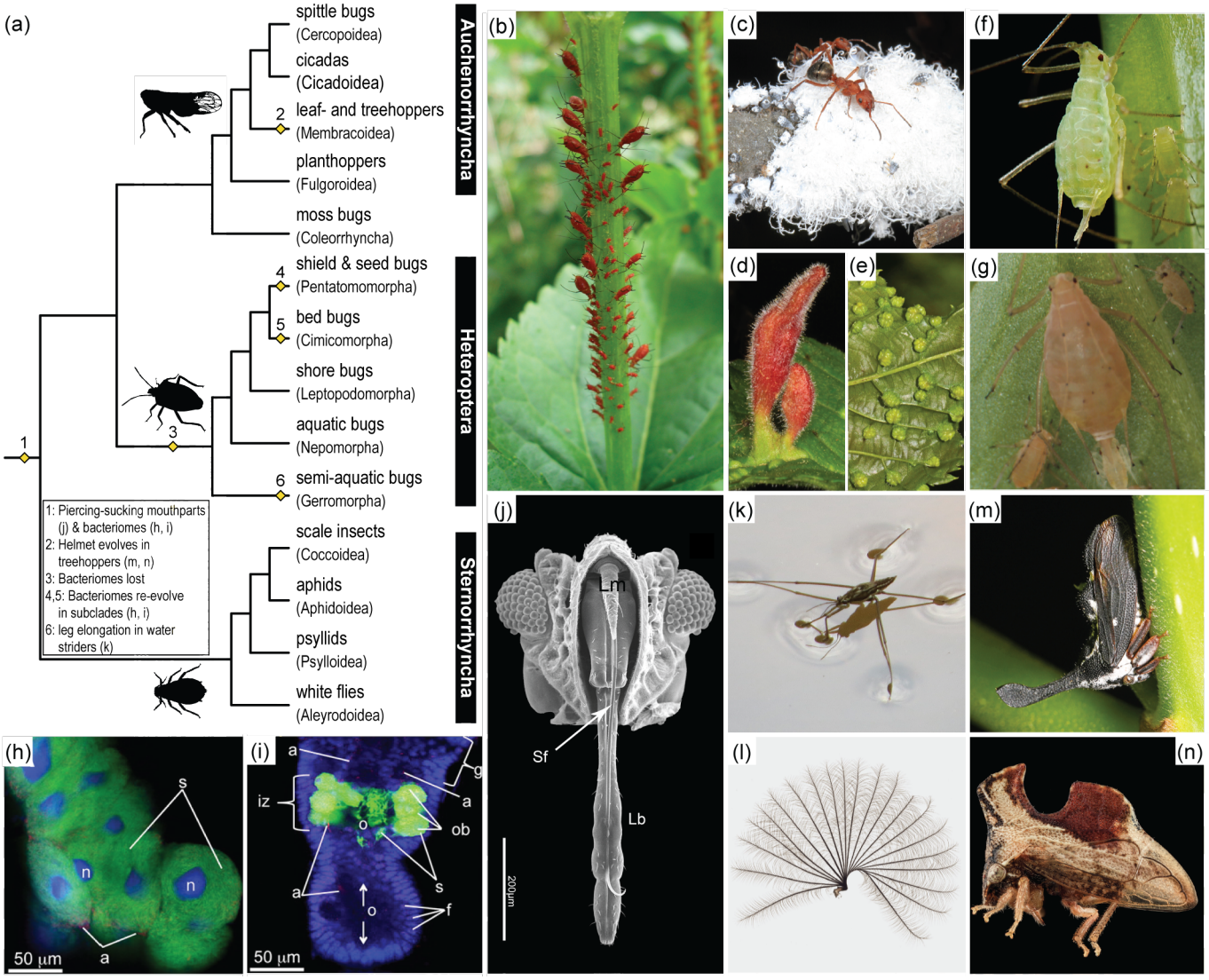
The association of new genes with new traits is an intriguing pattern, but it is unclear whether the pattern is evolutionarily significant. Several predictions related to this pattern are shown; opacity

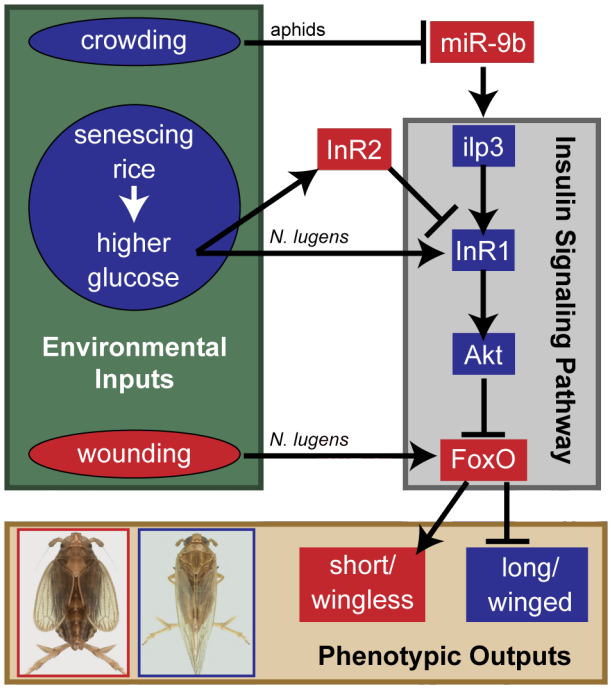
533 of circles reflects relative proportion of genes predicted to function in a given context; color
534 represents evolutionary age (purple–'control' genes with orthology across insects; red–'new'
535 hemipteran genes; orange–genes restricted to particular lineages within Hemiptera). For
536 example, are new genes more likely to function in the development of novel morphologies than
537 ancestral morphologies? A rigorous test would examine expression and function of a set of new
538 in both a novel trait and an evolutionarily older control trait, such as derived versus ancestral
539 features of hemipteran mouthparts (MP) (compare i versus ii). In that case, one might also
540 predict that their function in novel morphologies is more conserved (as shown by greater
541 variation across taxa in i versus ii). Second, taxon-restricted ('new') genes may be more likely
542 than control genes to function in the development of taxon-restricted ('new') traits (compare i
543 versus iii); the same contrast would not be expected in control traits (ii versus iv).

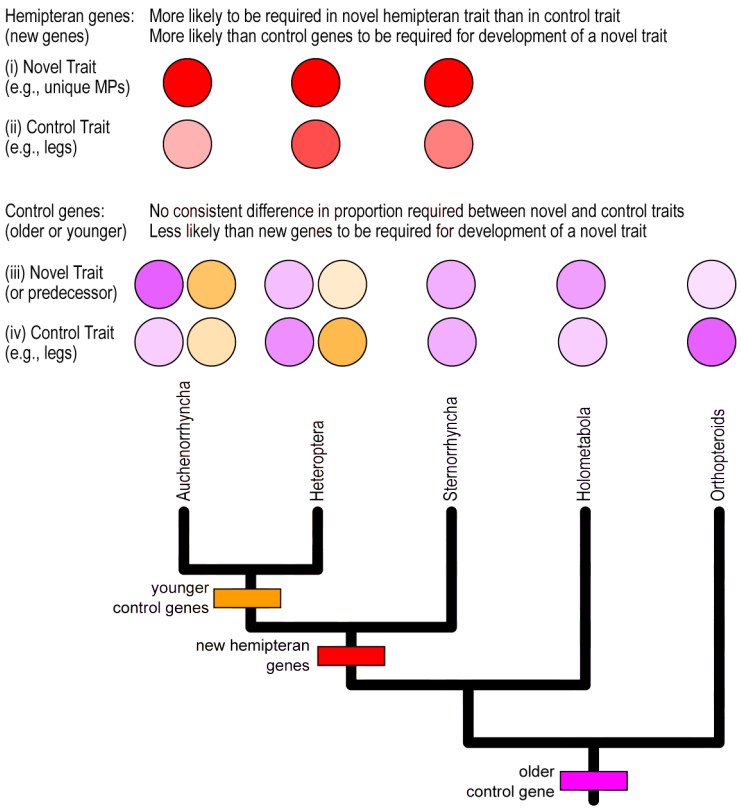
We are unaware of any conflicts of interest.



	G0	G1
O. fasciatus	92%	65%
N. lugens	0/2.7%	49%/3.2%
A. pisum	67%	35%


(a)
spittle bugs
(Cercopoidea)
cicadas
(Cicadoidea)
2
leaf- and treehoppers
(Membracoidea)
planthoppers
(Fulgoroidea)
moss bugs
(Coleorrhyncha)
4
shield & seed bugs
(Pentatomomorpha)
5
bed bugs
(Cimicomorpha)
shore bugs
(Leptopodomorpha)
3
aquatic bugs
(Nepomorpha)
1
6
semi-aquatic bugs
(Gerromorpha)
1: Piercing-sucking mouthparts (j) & bacteriomes (h, i)
2: Helmet evolves in treehoppers (m, n)
3: Bacteriomes lost
4,5: Bacteriomes re-evolve in subclades (h, i)
6: leg elongation in water striders (k)
scale insects
(Coccoidea)
aphids
(Aphidoidea)
psyllids
(Psylloidea)
white flies
(Aleyrodoidea)
Auchenorrhyncha
Heteroptera
Sternorrhyncha
(b)
(c)
(f)
(d)
(e)
(g)
(j)
Lm
Sf
Lb
200µm
(k)
(m)
(l)
(n)
(h)
s
n
n
a
50 µm
(i)
a
iz
o
a
ob
a
s
o
f
50 µm


crowding
aphids
miR-9b
senescing rice
higher glucose
InR2
N. lugens
ilp3
InR1
Akt
Insulin Signaling Pathway
Environmental Inputs
wounding
N. lugens
FoxO
short/ wingless
long/ winged
Phenotypic Outputs


Hemipteran genes:
(new genes)
More likely to be required in novel hemipteran trait than in control trait
More likely than control genes to be required for development of a novel trait
(i) Novel Trait
(e.g., unique MPs)
(ii) Control Trait
(e.g., legs)
Control genes:
(older or younger)
No consistent difference in proportion required between novel and control traits
Less likely than new genes to be required for development of a novel trait
(iii) Novel Trait
(or predecessor)
(iv) Control Trait
(e.g., legs)
Auchenorrhyncha
Heteroptera
Sternorrhyncha
Holometabola
Orthopteroids
younger
control genes
new hemipteran
genes
older
control gene