Molecular evolutionary trends and feeding ecology diversification in the Hemiptera, anchored by the milkweed bug genome

Kristen A. Panfilio[1, 2]*, Iris M. Vargas Jentzsch[1], Joshua B. Benoit[3], Deniz Erezyilmaz[4], Yuichiro Suzuki[5], Stefano Colella[6, 7], Hugh M. Robertson[8], Monica F. Poelchau[9], Robert M. Waterhouse[10, 11], Panagiotis Ioannidis[10], Matthew T. Weirauch[12], Daniel S.T. Hughes[13], Shwetha C. Murali[13, 14, 15], John H. Werren[16], Chris G.C. Jacobs[17, 18], Elizabeth J. Duncan[19, 20], David Armisén[21], Barbara M.I. Vreede[22], Patrice Baa-Puyoulet[6], Chloé S. Berger[21], Chun-che Chang[23], Hsu Chao[13], Mei-Ju M. Chen[9], Yen-Ta Chen[1], Christopher P. Childers[9], Ariel D. Chipman[22], Andrew G. Cridge[19], Antonin J.J. Crumière[21], Peter K. Dearden[19], Elise M. Didion[3], Huyen Dinh[13], HarshaVardhan Doddapaneni[13], Amanda Dolan[16, 24], Shannon Dugan[13], Cassandra G. Extavour[25, 26], Gérard Febvay[6], Markus Friedrich[27], Neta Ginzburg[22], Yi Han[13], Peter Heger[28], Thorsten Horn[1], Yi-min Hsiao[23], Emily C. Jennings[3], J. Spencer Johnston[29], Tamsin E. Jones[25], Jeffery W. Jones[27], Abderrahman Khila[21], Stefan Koelzer[1], Viera Kovacova[30], Megan Leask[19], Sandra L. Lee[13], Chien-Yueh Lee[9], Mackenzie R. Lovegrove[19], Hsiao-ling Lu[23], Yong Lu[31], Patricia J. Moore[32], Monica C. Munoz-Torres[33], Donna M. Muzny[13], Subba R. Palli[34], Nicolas Parisot[6], Leslie Pick[31], Megan Porter[35], Jiaxin Qu[13], Peter N. Refki[21, 36], Rose Richter[16, 37], Rolando Rivera Pomar[38], Andrew J. Rosendale[3], Siegfried Roth[1], Lena Sachs[1], M. Emília Santos[21], Jan Seibert[1], Essia Sghaier[21], Jayendra N. Shukla[34, 39], Richard J. Stancliffe[40], Olivia Tidswell[19, 41], Lucila Traverso[42], Maurijn van der Zee[17], Séverine Viala[21], Kim C. Worley[13], Evgeny M. Zdobnov[10], Richard A. Gibbs[13], Stephen Richards[13]*

* Correspondence: kristen.panfilio@alum.swarthmore.edu; stephenr@bcm.edu

The full list of author information is available at the end of the manuscript.

ABSTRACT

Background:
The Hemiptera (aphids, cicadas, and true bugs) are a key insect order whose members offer a close outgroup to the Holometabola, with high diversity within the order for feeding ecology and excellent experimental tractability for molecular genetics. Sequenced genomes have recently become available for hemipteran pest species such as phloem-feeding aphids and blood-feeding bed bugs. To complement and build upon these resources, we present the genome sequence and comparative analyses centered on the large milkweed bug, *Oncopeltus fasciatus*, a seed feeder of the family Lygaeidae.

Results:
The 926-Mb genome of *Oncopeltus* is relatively well represented by the current assembly and official gene set, which supports *Oncopeltus* as a fairly conservative hemipteran species for anchoring molecular comparisons. We use our genomic and RNA-seq data not only to characterize features of the protein-coding gene repertoire and perform isoform-specific RNAi, but also to elucidate patterns of molecular evolution and physiology. We find ongoing, lineage-specific expansion and diversification of repressive C2H2 zinc finger proteins and of intron gain and turnover in the Hemiptera. These analyses also weigh the relative importance of lineage and genome size as predictors of gene structure evolution in insects. Furthermore, we identify enzymatic gains and losses that correlate with hemipteran feeding biology, particularly for reductions in chemoreceptor family size and loss of metabolic reactions within species with derived, fluid-nutrition feeding modes.

Conclusions:
With the milkweed bug genome, for the first time we have a critical mass of sequenced species representing a hemimetabolous insect order, substantially improving the diversity of insect genomics beyond holometabolans such as flies and ants. We use this addition to define commonalities among the Hemiptera and then delve into how hemipteran species' genomes reflect their feeding ecology types. Our novel and detailed analyses integrate global and rigorous manual approaches, generating hypotheses and identifying specific sets of genes for future investigation. Given *Oncopeltus*'s strength as an experimental research model, we take particular care to evaluate the sequence resources presented here, augmenting its foundation for molecular research and highlighting potentially general considerations exemplified in the assembly and annotation of this medium-sized genome.

Keywords:
Phytophagy; Transcription Factors; Gene Structure; Lateral Gene Transfer; RNAi; Gene family evolution; Evolution of Development

BACKGROUND

In the past few years, the number of animals with sequenced genomes has increased dramatically, and there are now over 100 insect species with assembled and annotated genomes [1]. However, the majority belong to the Holometabola (*e.g.*, flies, beetles, wasps, butterflies), the group characterized by a biphasic life history with distinct larval and adult phases separated by a dramatic metamorphosis during a pupal stage. With fewer than half of all orders, the Holometabola represent only a fraction of the full morphological and ecological diversity across the Insecta. This imbalance in genomic resources limits the exploration of this diversity, including the environmental and developmental requirements of a hemimetabolous life style with a progression of flightless nymphal (juvenile) instars. Addressing this paucity, we report here comparative analyses based on genome sequencing of the large milkweed bug, *Oncopeltus fasciatus*, as a hemimetabolous representative of the larger diversity of insects.

The Hemiptera, the order to which *Oncopeltus* belongs, comprise the most species-rich hemimetabolous order and a close outgroup to the Holometabola as part of the hemipteroid assemblage (or Acercaria), with the Thysanoptera as a sister order and the Psocodea also traditionally included in this clade [2, 3]. All Hemiptera share the same piercing and sucking mouthpart anatomy [4], yet they have diversified to exploit food sources ranging from seeds and plant tissues (phytophagy) to phloem sap (mucivory) and mammalian blood (hematophagy). For this reason, many hemipterans are agricultural pests or human disease vectors, and genome sequencing efforts to date have focused on these species (Fig. 1), including phloem-feeding aphids [5-7], psyllids [8], and planthoppers [9], and the hematophagous kissing bug, *Rhodnius prolixus* [10], a vector of Chagas disease, and bed bug, *Cimex lectularius* [11, 12]. Building on transcriptomic data, genome projects are also in progress for other pest species within the same infraorder as *Oncopeltus*, such as the stink bug *Halyomorpha halys* [13, 14].

In this context, *Oncopeltus* represents a relatively benign species with conservative life history traits, affording a baseline against which other species can be compared. As a seed feeder, *Oncopeltus* has not undergone the marked life style changes that are associated with fluid feeding (mucivory or hematophagy), including dependence on endosymbiotic bacteria to provide needed complements lacking in the diet. For example, in the pea aphid, *Acyrthosiphon pisum*, its obligate endosymbiont, *Buchnera aphidicola,* provides essential amino acids and vitamins: previous analysis of the two genomes revealed a complementation of the two organisms' amino acid metabolism systems [5, 15]. Similarly, although hematophagy arose independently in *Rhodnius* and *Cimex* [16], their respective endosymbionts, *Rhodococcus rhodnii* and *Wolbachia*, provide vitamins lacking in the blood diet [17]. In contrast, the seed-feeding subfamily Lygaeinae, including *Oncopeltus*, is notable for the absence of prominent endosymbiotic anatomy: these bugs lack not only the midgut crypts that typically house bacteria but also the bacteriomes and endosymbiotic balls seen even in other Lygaeidae [18].

Nonetheless, as the native food source of *Oncopeltus* is the milkweed plant, its own feeding biology has a number of interesting implications associated with detoxification and sequestration of cardenolide compounds, including the bright red-

orange aposematic (warning) coloration seen in *Oncopeltus* embryos, nymphs, and adults [19, 20]. Thus, diet, metabolism, and body pigmentation are functionally linked biological features for which one may expect changes in gene repertoires to reflect diversity across species of the same order, and the Hemiptera provide an excellent opportunity to explore this.

Furthermore, *Oncopeltus* has been an established laboratory model organism for over 60 years, with a rich experimental tradition in a wide range of studies from physiology and development to evolutionary ecology [20-22]. It is among the few experimentally tractable hemimetabolous insect species, and it is amenable to a range of molecular techniques (*e.g.*, [23-25]). In fact, it was one of the first insect species to be functionally investigated by RNA interference (RNAi, [26]). RNAi in *Oncopeltus* is highly effective across different life history stages, which has led to a resurgence of experimental work over the past fifteen years, with a particular focus on the evolution of developmentally important regulatory genes (reviewed in [22]).

Focusing on these two avenues – feeding biology diversity within the Hemiptera and *Oncopeltus* as a research model for macroevolutionary genetics – we present here key insights derived from a combination of global comparative genomics and detailed computational analyses supported by extensive manual curation, empirical data for gene expression, sequence validation, and new isoform-specific RNAi. Namely, we identify sets of genes with potentially restricted life history expression in *Oncopeltus* and that are unique to the Hemiptera, clarify evolutionary patterns of zinc finger protein expansion, identify predictors of insect gene structure, and identify lateral gene transfer and amino acid metabolism features that correlate with feeding biology.

RESULTS AND DISCUSSION

The genome and its assembly

Oncopeltus fasciatus has a diploid chromosome number (2*n*) of 16, comprised of seven autosomal pairs and two sex chromosomes with the XX/XY sex determination system [27, 28]. To analyze this genetic resource, we sequenced and assembled the genome using next-generation sequencing approaches (Table 1; see also Methods and Supplemental Notes Sections 1-4). We measure the genome size to be 923 Mb in females and 928 Mb in males based on flow cytometry data (see also Supplemental Note 2.1.a), such that the assembly contains 84% of the expected sequence in assembled contigs, which is comparable to that of other recent, medium-sized insect genomes [11, 29]. However, our analyses of the *k*-mer frequency distribution in raw sequencing reads yielded ambiguous estimates of genome size and heterozygosity rate, which is suggestive of both high heterozygosity and high repetitive content ([30], see also Supplemental Note 2.1.b). Consistent with this, in further analyses we obtained high estimates of repetitive content (see below), which would imply a large proportion of potentially redundant sequence and possible misassembly within contigs of the current assembly. This phenomenon may be increasingly relevant as comparative genomics based on short read sequencing extends to additional insect species with genomes in the 1-Gb range.

As template DNA was prepared from dissected adults from which gut material was removed, the resulting assembly is essentially free of contamination, with only five small scaffolds with high bacterial homology (each to a different, partial bacterial genome, see also Supplemental Note 2.2), which either represent trace bacterial contamination or lateral gene transfers that have not assembled to flanking eukaryotic DNA, making their confirmation in the current assembly difficult.

Table 1. *Oncopeltus fasciatus* genome metrics.

Feature	Value	
2*n* chromosomes	16	
Genome size	926 Mb (mean between males and females)	
Assembly size	1,099 Mb (contigs only: 774 Mb)	
Coverage	106.9× raw coverage, 83.7% of reads in final assembly	
Contig N50	4,047 bp	
Scaffold N50	340.0 kb	
# Scaffolds	17,222	
GC content	genome: 32.7%, protein-coding sequence (OGS v1.2): 42%	
OGS v1.1 (curated fraction)	19,690 models[1] (1,426 models, 7.2%)	19,465 genes (1,201 genes, 6.2%)
OGS v1.2 (curated fraction)	19,809 models[1] (1,697 models, 8.7%)	19,616 genes (1,518 genes, 7.7%)

[1] Individual genes may be represented by multiple models in cases of curated alternative isoforms or if the gene is split across scaffolds.

The official gene set and conserved gene linkage

The official gene set (OGS) was generated by automatic annotation followed by manual curation in a large-scale effort by the research community (see also Supplemental Notes Sections 3-4). Curation revised automatic annotation models, added alternative isoforms and *de novo* models, and documented multiple discrete models for genes whose exons were split across scaffolds. We found that automatic predictions were somewhat conservative for hemipteran gene structure (see below), and manual curation primarily resulted in larger gene loci as exons were added and/or extended, including merging discrete automatic models (see also Supplemental Note 4, Table S4.4). The OGS v1.1 was generated for global, pipeline analyses to characterize the gene repertoire. The latest version, OGS v1.2, represents a minor update, primarily for the addition of chemoreceptor genes of the ionotropic and odorant receptor classes and curation of genes encoding metabolic enzymes. Altogether, the research community curated 1,697 gene models (8.7% of OGS v1.2), including 316 *de novo* models (see also Table S4.1). Reflecting the primary research interests of the community (see also Supplemental Notes Section 5), the majority of curated models are for genes encoding cuticular proteins (11%), chemoreceptors (19%), and developmental regulators such as transcription factors and signaling pathway components (40%, including the BMP/TGF-β, Toll/NF-κB, Notch, Hedgehog, Torso RTK, and Wnt pathways).

In addition to assessing gene model quality, manual curation of genes whose orthologs are expected to occur in syntenic clusters also validates assembly scaffolding. Complete loci could be found for single orthologues of all Hox cluster genes, where *Hox3*/*zen* and *Hox4*/*Dfd* are linked in the current assembly and have ≥99.9% nucleotide identity with experimentally validated sequences ([31-33], Supplemental Note 5.1.b). Conserved linkage was also confirmed for the homeobox genes of the Iroquois complex, the Wnt ligands *wingless* and *wnt10*, and two linked pairs from the Runt transcription factor complex (Supplemental Notes 5.1.a, 5.1.c, 5.1.i, 5.1.j). Further evidence for correct scaffold assembly comes from the curation of large, multi-exonic loci. For example, the cell polarity and cytoskeletal regulator encoded by the conserved *furry* gene includes 47 exons spanning a 437-kb locus, which were all correctly assembled on a single scaffold.

Transcriptomic resources and gene expression profiles across the milkweed bug life cycle

To augment published transcriptomic resources [34, 35], we sequenced three different post-embryonic samples ("i5K" dataset, see Methods). We then compared the OGS to the resulting *de novo* transcriptome and to a previously published embryonic and maternal (ovary) transcriptome ("454" pyrosequencing dataset, [34]). Our OGS is quite comprehensive, containing 90% of transcripts from each transcriptomic dataset and an additional 3,146 models (16% of OGS: Fig. 2a). Among the additional models, 274 (9%) were manually validated, including 163 *de novo* models for odorant and gustatory receptors. These gene classes are known for lineage-specific expansions and highly tissue- and stage-specific expression, with usually only one receptor expressed per sensory neuron ([36, 37], and see below).

Furthermore, the OGS does a good job of "mopping up" partial and unidentified 454 transcripts. We could substantially improve orthologous gene discovery by mapping the 454 transcripts to the OGS (blastn, $e<10^{-9}$), nearly trebling the proportion of transcripts with an assigned gene model or homology compared to the original study (from 9% to 26%). This included 10,130 transcripts that primarily mapped to UTRs and could not have been identified by coding sequence homology, such as the 654-bp transcript for the *Oncopeltus brinker* ortholog, which encodes a putative inhibitor of the BMP pathway ([38], see also Supplemental Note 5.1.f), and four unassembled transcripts each from the 3′ UTRs of the enzyme-encoding genes *CTP synthase* and *roquin*. At the same time, the transcriptomes provided expression support for the identification of multiple isoforms in the OGS. For example, we could confirm previously described isoforms for the germline determinant encoded by *nanos* [34]. Where assembly limitations curtailed OGS gene models, full-length transcripts are represented in the transcriptomes, such as for the ecdysis regulator CCAP-R [39] and the chromatin linker Histone H1.

We then took advantage of our stage-specific RNA datasets to provide an initial survey of gene expression profiles across biological samples and across the life cycle. Most OGS gene models have expression support (91% of 19,690), with 74% expressed broadly in at least three of four samples (Fig. 2b). The inclusion of a fifth dataset from a published adult library [35] provided only a 1% gain in expression support (218 gene models), indicating that with the current study the expression data volume for *Oncopeltus* is quite complete. At the same time, direct comparison of the three adult samples suggests that the published adult dataset of unspecified sex is probably male, as it shares 4.6× more expressed genes with our male than our female sample.

As these data derive from limited biological sampling, we remain cautious about true stage specificity and do not quantify expression levels. We do, however, note that most genes with stage-restricted expression are in sets involving our male sample (Fig. 2b: male-only or male and nymph), although this sample does not contain more reads or more expressed genes. Furthermore, we also find stage-specific patterns for some of our most abundant curated gene classes. Gustatory receptor (GR) genes show noticeable restriction to the adult male and published adult (probable male) samples (n= 169 GRs: 40% no expression, 27% only expressed in these two samples), with half of these expressed in both biological replicates (52%). Interestingly, the nymphal sample is enriched for genes encoding structural cuticular proteins (94%, which is >56% more than any other sample). This likely reflects the ongoing molting cycles, with their cyclical upregulation of cuticular gene synthesis [40], that are experienced by the different instars and molt cycle stages of individuals pooled in this sample.

Protein orthology and hemipteran copy number comparisons

To further assay protein-coding gene content, we then compared *Oncopeltus* with eleven other arthropod species. A phylogeny based on single copy orthologs correctly reconstructs the hemipteran and holometabolan clades' topologies (Fig. 3a, compare with Fig. 1a), although larger-scale insect relationships remain challenging [3]. In expanding this to the Benchmarking Universal Single-Copy Orthologs (BUSCO, [41]) dataset of 2,675 Arthropoda genes, we also found that most BUSCO genes are

present in the *Oncopeltus* OGS, although with additional genes identified on genomic scaffolds but not yet incorporated into the gene set (see also Supplemental Note 6.1). We next categorized all proteins by conservation in global, clustering-based orthology analyses [42]. As in most species, half of *Oncopeltus* proteins (51%) falls within the top three conservation levels (Fig. 3a). Moreover, 98% of all *Oncopeltus* protein-coding genes has homology, expression, and/or curation support (Fig. 3b), including support for 80% of proteins without homology, such as a few species-specific chemoreceptors and antimicrobial peptides (see also Supplemental Note 5.1.h), while some unsupported models may be split or partial. Overall, we estimate that the *Oncopeltus* protein repertoire is comparable to that of other insects in size and degree of conservation.

In contrast, the pea aphid, *Acyrthosiphon pisum*, is a notable outlier even among fellow Hemiptera, where we provide a side-by-side comparison with *Oncopeltus* as well as the recently-sequenced kissing bug, *Rhodnius prolixus* [10], and bed bug, *Cimex lectularius* [11, 12]. The pea aphid is striking for its long branch in phylogenetic comparisons and for its large protein-coding gene content with low conservation (Fig. 3a), consistent with the observation of numerous lineage-specific duplications [5]. As the first hemipteran to have its genome sequenced, the pea aphid has often been used to boost taxonomic sampling in phylogenomic comparisons (*e.g.*, [29]). However, the pea aphid may not be the best representative, and as more hemipteran genomes are sequenced, other species now offer less derived alternatives.

Compared to the pea aphid [43], *Oncopeltus* is more conservative in both presence and copy number for several signaling pathway components. In contrast to gene absences described for the pea aphid, *Oncopeltus* retains orthologs of the EGF pathway component *sprouty*, the BMP receptor *wishful thinking*, and the hormone nuclear receptor *Hr96* (see also Supplemental Note 5.1.e). Similarly, whereas multiple copies were reported for the pea aphid, we find a single *Oncopeltus* ortholog for the BMP pathway components *decapentaplegic* and *Medea* and the Wnt pathway intracellular regulator encoded by *shaggy/GSK-3*, albeit with five potential isoforms of the latter (see also Supplemental Notes 5.1.f, 5.1.j). Duplications of miRNA and piRNA gene silencing components likewise seem to be restricted to the pea aphid compared to other hemipterans – including other aphid species ([44], see also Supplemental Note 5.4.a). However, our survey of *Oncopeltus* and several other hemimetabolous species reveals evidence for frequent parallel duplications of the Wnt pathway component *armadillo/β-catenin*, yet without the sequence and functional divergence previously observed independently in the pea aphid and *Tribolium* ([45], see also Supplemental Note 5.1.j). Curiously, *Oncopeltus* appears to encode fewer histone loci than any other arthropod genome and yet exhibits a similar, but possibly independent, pattern of duplications of histone acetyltransferases to those previously identified in *Cimex* and the pea aphid (see also Supplemental Note 5.4.c).

On the other hand, we documented several notable *Oncopeltus*-specific duplications. Whereas two copies of the BMP transducer *Mad* were reported in the pea aphid [43], we find evidence for three paralogs in *Oncopeltus*, where two of these genes occur in tandem and may reflect a particularly recent duplication (see also Supplemental Note 5.1.f). Similarly, a tandem duplication of *wnt8* appears to be unique to *Oncopeltus* (see also Supplemental Note 5.1.j). More striking is the

identification of six potential paralogs of *cactus*, a member of the Toll/NF-κB signaling pathway for innate immunity, whereas the bed bug and kissing bug each retain only a single copy ([46], see also Supplemental Note 5.1.g).

We then took advantage of broader comparative datasets [42] to identify lineage-specific features of the Hemiptera. In other words, what makes a bug a bug in terms of protein-coding genes? To address this, we partitioned an orthology analysis of 64 insect species into three broad taxonomic groups (Fig. 3c). Highlights for the Hemiptera, which are further corroborated in an updated dataset with 116 insect species (OrthoDB v.9.1, [1]), fall into two classes. The first class contains potentially new genes that show no homology outside the Hemiptera. We identified three such instances with orthologous protein members present in at least four hemipterans, and where no conserved functional domains were recognized. Interpretation of these intriguing "uncharacterized proteins" will have to await direct experimental analyses, for which the Hemiptera in general are particularly amenable (*e.g.*, [47-50]). The second class comprises proteins with recognized functional domains and homologs in other insects, but where evolutionary divergence has led to hemipteran-specific subfamilies. For example, one protein orthology group ("orthogroup" EOG090W0V4B) is comprised of a heteropteran-specific cytochrome P450 (CYP) enzyme that in *Oncopeltus* is expressed in all life history stages. The expansion of this protein family is associated with a species' potential scope for insecticide resistance, as specific P450s can confer resistance to specific chemicals (*e.g.,* [51, 52]; see also Supplemental Notes 5.3.b, 5.3.c). Hence, the identification of lineage-specific CYP enzymes can suggest potential targets for integrated pest management approaches.

Transcription factor repertoires and homeobox gene evolution

Having explored the global protein repertoire, we next focused specifically on transcription factors (TFs), which comprise a major class of proteins that has been extensively studied in *Oncopeltus*. This is a class of key regulators of development whose functions can diverge substantially during evolution and for which RNAi-based experimental investigations have been particularly fruitful in the milkweed bug (*e.g.*, [31, 32, 53-55], see also Supplemental Notes 5.1.a-e).

To systematically evaluate the *Oncopeltus* TF repertoire, we used a pipeline to scan all predicted proteins and assign them to TF families, including orthology assignments in cases where DNA binding motifs could be predicted (see Methods, [56]). We identified 762 putative TFs in *Oncopeltus*, which is similar to other insects of diverse orders for total TF count and for the size of each TF family (Fig. 4a: note that the heatmap also reflects the large, duplicated repertoire in the pea aphid; see also Tables S6.2-6.4).

We were able to infer DNA binding motifs for 25% (n=189) of *Oncopeltus* TFs, mostly based on data from *D. melanogaster* (121 TFs) but also from distantly related taxa such as mammals (56 TFs). Such high conservation is further reflected in the fact that most proteins within several large TF families have inferred motifs and therefore explicit orthology assignments, including for the homeodomain (53 of 85, 62%), basic helix-loop-helix (bHLH, 35 of 45, 78%), and forkhead box (16 of 17, 94%) families. In contrast, most C2H2 zinc finger proteins lack orthology assignment

(only 22 of 360, 6%). Across species, the homeodomain and C2H2 zinc finger proteins are the two largest TF superfamilies (Fig. 4a). Given their very different rates of orthology assignment in *Oncopeltus*, we probed further into the pipeline predictions and the patterns of evolutionary diversification of these proteins.

The number of homeodomain proteins identified by the pipeline displays a narrow normal distribution across species (Fig. 4b, mean ± standard deviation: 97 ± 9), consistent with a highly conserved, slowly evolving protein family. Supporting this, many *Oncopeltus* homeodomain proteins that were manually curated also received a clear orthology assignment (Fig. 4c: pink), with only four exceptions (Fig. 4c: yellow). Only one case suggests a limitation of a pipeline that is not specifically tuned to hemipteran proteins (Goosecoid), while an incomplete gene model received homeodomain classification but without explicit orthology assignment (Distal-less). Manual curation of other partial or split models identified a further 11 genes encoding homeodomains, bringing the actual tally in *Oncopeltus* to 96, which is comparable to the mean across species. Overall, we find the TF pipeline results to be a robust and reasonably comprehensive representation of these gene classes in *Oncopeltus*.

These analyses also uncovered a correction to the published *Oncopeltus* literature for the key developmental patterning proteins encoded by the closely related paralogs *engrailed* and *invected*. These genes arose from an ancient tandem duplication prior to the hexapod radiation, where their tail-to-tail orientation enables ongoing gene conversion [57], making orthology discrimination particularly challenging. For *Oncopeltus*, we find that the genes also occur in a tail-to-tail orientation and that *invected* retains a diagnostic alternative exon [57]. These new genomic and expression data reveal that the *Oncopeltus* gene used as the purported *engrailed* ortholog in previous developmental studies (*e.g.*, [53, 58-61]) is in fact *invected* (see also Supplemental Note 5.1.a).

Independent expansions of C2H2 zinc fingers within the Hemiptera

Unlike homeodomain proteins, C2H2 zinc finger (C2H2-ZF) repertoires are prominent for their large family size and variability throughout the animal kingdom [62], and this is further supported by our current analysis in insects. With >350 C2H2-ZFs, *Oncopeltus*, the pea aphid, the termite, and several mosquito species have 1.5× more members than the insect median (Fig. 4b). This is nearly half of all *Oncopeltus* TFs. While the expansion in mosquitoes could have a single origin after the Culicinae diverged from the Anophelinae, the distribution in the Hemiptera, where *Cimex* has only 227 C2H2-ZFs, suggests that independent expansions occurred in *Oncopeltus* and the pea aphid. Prior to the sequencing of other hemipteran genomes, the pea aphid's large C2H2-ZF repertoire was attributed to the expansion of a novel subfamily, APEZ, also referred to as zinc finger 271-like [43].

In fact, manual curation in *Oncopeltus* confirms the presence of a subfamily with similar characteristics to APEZ (Fig. 4c: 42% of all C2H2-ZFs were curated, including 38% of those without orthology assignment, yellow). Specifically, in *Oncopeltus* we find >115 proteins of the ZF271 class that are characterized by numerous tandem repeats of the C2H2-ZF domain and its penta-peptide linker, with 3-45 repeats per protein.

However, at both the gene and protein levels we find evidence for ongoing evolutionary diversification of the *Oncopeltus* ZF271-like subfamily. A number of *Oncopeltus* ZF271-like genes occur in tandem clusters of 4-8 genes, suggesting recent duplication events. Yet, at the same time, gene structure (number and size of exons) is not shared between genes within clusters, and we identified a number of probable ZF271-like pseudogenes whose open reading frames have become disrupted – consistent with high turnover. At the domain level, *Oncopeltus* ZF271-like proteins differ in the sequence and length of the zinc finger domains amongst themselves and compared to aphid proteins (WebLogo analysis [63]), similar to zinc finger array shuffling seen in humans [64]. Furthermore, whole-protein phylogenetic analysis supports independent, rapid expansions in the pea aphid and *Oncopeltus* (Fig. 4d).

Clustered zinc finger gene expansion has long been recognized in mammals, with evidence for strong positive selection to increase both the number and diversity of zinc finger domains per protein as well as the total number of proteins [65]. This was initially found to reflect an arms-race dynamic of co-evolution between selfish transposable elements and the C2H2-ZF proteins that would repress them [66]. In vertebrates, these C2H2-ZF proteins bind to the promoters of transposable elements via their zinc finger arrays and use their Krüppel-associated box (KRAB) domain to bind the chromatin-remodeling co-repressor KAP-1, which in turn recruits methyltransferases and deacetylases that silence the targeted promoter [67].

Insects do not have a direct ortholog of vertebrate KAP-1 (see also Supplemental Note 5.4.d), and neither the aphid nor *Oncopeltus* ZF271-like subfamilies possess a KRAB domain or any other domain besides the zinc finger arrays. However, close molecular outgroups to this ZF271-like subfamily include the developmental repressor Krüppel [68] and the insulator protein CTCF [69] (data not shown). Like these outgroups, the *Oncopeltus* ZF271-like genes are strongly expressed: 98% have expression support, with 86% expressed in at least three different life history stages (Fig. 2b). Thus, the insect ZF271-like proteins may also play prominent roles in repressive DNA binding. Indeed, we find evidence for a functional methylation system in *Oncopeltus* (see also Supplemental Note 5.4.c), like the pea aphid, which would provide a means of gene silencing by chromatin remodeling, albeit via mediators other than KAP-1.

However, an arms race model need not be the selective pressure that favors insect ZF271-like family expansions. Recent analyses in vertebrates identified sophisticated, additional regulatory potential by C2H2-ZF proteins, building upon original transposable element binding for new, lineage-specific and even positive gene regulation roles [64, 70, 71]. Moreover, although *Cimex* has half as many long terminal repeat (LTR) repetitive elements as *Oncopeltus* and the pea aphid, they constitute only a minor fraction of these species' transposable elements, and overall we do not find a correlation between relative or absolute repetitive content and ZF271-like family expansion within the Hemiptera (Fig. 5, and see below).

Proportional repeat content across hemipterans

With the aim of reducing assembly fragmentation and to obtain a better picture of repeat content, we performed low coverage, long read PacBio sequencing in *Oncopeltus* (see also Supplemental Note 2.3). Using PacBio reads in a gap-filling

assay on the Illumina assembly raised the total detected repetitive content from 25% to 32%, while repeat estimations based on simultaneous assessment of Illumina and PacBio reads nearly doubled this value to 58%. As expected, the capacity to identify repeats is strongly dependent on assembly quality and sequencing technology, with the *Oncopeltus* repetitive content underrepresented in the current (Illumina-only) assembly. Furthermore, as increasing genome size compounds the challenge of assembling repeats, the repeat content of the current assembly is lower than in species with smaller genome sizes (Fig. 5a, with the sole exception of the honey bee), and we therefore used our gap-filled dataset for further repeat profile comparisons.

To allow for direct comparisons among hemipterans, we also performed our RepeatModeler analysis on the bed bug and pea aphid assemblies. In these analyses, 36% and 31% of the respective assemblies were covered by repeats, similar to the gap-filled value of 32% in *Oncopeltus*. Nevertheless, given the smaller sizes of these species' assemblies – 651 Mb in the bed bug and 542 Mb in the pea aphid – the absolute repeat content is much higher in *Oncopeltus* (Fig. 5b**)**. Excluding unknown repeats, the most abundant transposable elements in *Oncopeltus* are LINE retrotransposons, covering 10% of the assembly (see also Table S2.5). This is also the case in the bed bug (12%), while in the pea aphid DNA transposons with terminal inverted repeats (TIRs) are the most abundant (2% of the assembly identified here, and 4% reported from manual curation in the pea aphid genome paper, [5]). Across species, the remaining repeat categories appear to grow proportionally with assembly size, except for simple repeats, which were the category with the largest relative increase in size after gap-filling in *Oncopeltus* (see also Supplemental Note 2.3). However, given the mix of sequence data types (Illumina only in the bed bug [11], Sanger in the pea aphid [5]), these patterns should be treated as hypotheses for future testing, until the assembly of repetitive regions becomes more feasible.

Lineage and genome size-related trends in insect gene structure

During manual curation, we noticed that *Oncopeltus* genes were often comprised of many, small exons. Furthermore, sequence conservation among the Hemiptera supported terminal coding sequence exons that were small and separated from the rest of the gene model by large introns. To explore patterns of gene structure across the insects, we undertook a broader comparative analysis. We find that both lineage and genome size can serve as predictors of gene structure.

Firstly, we created a high quality ("gold standard") dataset of 30 functionally diverse, large genes whose manual curation could reasonably ensure complete gene models across seven species from four insect orders (Fig. 6a; see also Supplemental Note 6.3). Most species encode the same total number of amino acids for these conserved proteins, with the thrips *Frankliniella occidentalis* and *Drosophila* being notable exceptions with larger proteins (Fig. 6a: blue plot line). However, the means of encoding this information differs between lineages, with hemipteroid orthologs comprised of twice as many exons as their holometabolous counterparts (Fig. 6a: orange plot line). Thus, there is an inverse correlation between exon number and exon size (Fig. 6a: orange vs. red plot lines). This analysis corroborates and extends previous probabilistic estimates of intron density, where the pea aphid as a sole hemipteran representative had the highest intron density of ten insect species [72].

To test these trends, we next expanded our analysis to all manually curated exons in two species from each of three orders, including the Mediterranean fruit fly, *Ceratitis captitata* [73], as a second dipteran alongside *Drosophila melanogaster*. Here, we expect that curated exon sizes are accurate, without the need to assume that entire gene models are complete. This large dataset supports our original findings, with bugs having small exons, and with both the median and Q3 quartile reflecting larger exon sizes in beetles and flies (Fig. 6b). Notably, the median and median absolute deviation are highly similar between species pairs within the Hemiptera and Coleoptera, irrespective of sample size. Meanwhile, the different exon metrics between *Ceratitis* and *Drosophila* suggest that the large protein sizes we initially observed in *Drosophila* (Fig. 6a: blue plot line) are a general but drosophilid-specific, rather than dipteran-wide, feature.

Does the high exon count in the Hemiptera reflect an ancient, conserved increase at the base of this lineage, or ongoing remodeling of gene structure with high turnover? To assess the exact nature of evolutionary changes, we annotated intron positions within multiple sequence alignments of selected proteins and plotted gains and losses onto the phylogeny, providing a total sample of 165 evolutionary changes at 148 discrete splice sites (Fig. 7; see also Supplemental Note 6.3 for gene selection and method). These data reveal several major correlates with intron gain or loss. The bases of both the hemipteroid and hemipteran radiations show the largest gains, while most losses occur in the dipteran lineage (Fig. 7: orange and purple shading, respectively). Furthermore, we find progressive gains across the hemipteroid nodes, and it is only in these species that we additionally find species-specific splice changes for the highly conserved *epimerase* gene (Fig. 7: orange outline). Thus, we find evidence for both ancient intron gain and ongoing gene structure remodeling in this lineage.

Surprisingly, both *hemocytin* and *epimerase* – our exemplar genes with many (up to 74) and very few exons (3-8 per species), respectively – show independent losses of the same splice sites in *Drosophila* and *Tribolium*. One feature these species share is a genome size 2.4-6.0× smaller than all other species examined here (Fig. 7: red shading). Pairwise comparisons within orders also support this trend, as the beetle and fly species with larger genomes exhibit species-specific gains compared to intron loss in their sister taxa (Fig. 7: red outlines). Thus, while lineage is a stronger predictor of gene structure evolution (the coleopteran and dipteran species include one each with a big or small genome and yet have highly similar metrics in Fig. 6b), genome size seems to positively correlate with intron number (*e.g.,* the common dipteran ancestor lost introns before *Ceratitis*, with a larger genome, experienced subsequent gains: Fig. 7). A global computational analysis over longer evolutionary distances also supports a link between genome size and intron number within arthropods, but where chelicerates and insects may experience different rates of evolutionary change in these features [74]. As new insect species' genomes are sequenced, gene structure expectations at the ordinal level can help customize parameters for automatic gene annotation, while it will be interesting to see if the correlation with genome size is borne out in other taxa.

The selective pressures and mechanisms of intron gain in the Hemiptera will be a challenge to uncover. While median exon size (Fig. 6b) could reflect species-specific nucleosome sizes [75, 76], this does not account for the fact that most

hemipteran exons do not occur in multiples greater than a single nucleosome. Given gaps in draft genome assemblies, we remain cautious about interpreting (large) intron lengths but note that many hemipteran introns are too small to have harbored a functional transposase gene (*e.g.*, median intron size of 429 bp, n=69 introns in *hemocytin* in *Cimex*). Such small introns could be consistent with proliferation of non-autonomous short interspersed nuclear elements (SINEs), although as highly divergent non-coding elements their characterization in insects would require curated SINE libraries comparable to those generated for vertebrates and plants [75, 76]. Meanwhile, it appears that hemipteran open reading frames ≥160 bp are generally prevented by numerous in-frame stop codons just after the donor splice site. Most stop codons are encoded by the triplet TAA in both *Oncopeltus* and *Cimex* (data not shown), although these species' genomes are not particularly AT-rich (Table 1).

Even if introns are small, having gene loci comprised of numerous introns and exons adds to the cost of gene expression in terms of both transcription duration and mRNA processing. One could argue that a gene like *hemocytin*, which encodes a clotting agent, would require rapid expression in the case of wounding – a common occurrence in adult *Cimex* females due to the traumatic insemination method of reproduction [11]. Thus, as our molecular understanding of comparative insect and particularly hemipteran biology deepens, we will need to increasingly consider how life history traits are manifest in genomic signatures at the structural level (*e.g.*, Figs. 5-7), as well as in terms of protein repertoires (Figs. 3-4).

Expansion after a novel lateral gene transfer (LGT) event in phytophagous bugs

In addition to the need for cuticle repair, traumatic insemination may be responsible for the numerous LGT events predicted in the bed bug [11]. In contrast, the same pipeline analyses [77] followed by manual curation predicted very few LGTs in *Oncopeltus*, which lacks this unusual mating behavior. Here, we have identified 11 strong LGT candidates, and we confirmed the incorporation of bacterial DNA into the milkweed bug genome for all five candidates chosen for empirical testing (see also Table S2.4). Curiously, we find several LGTs potentially involved in bacterial or plant cell wall metabolism that were acquired from different bacterial sources at different times during hemipteran lineage evolution, including two distinct LGTs that are unique to *Oncopeltus* and implicated in the synthesis of peptidoglycan, a bacterial cell wall constituent (see also Supplemental Note 2.2).

Conversely, two further validated LGT candidates encode enzymes rather known for their roles in degradation of bacterial cell walls: we find two strongly expressed, paralogous copies in *Oncopeltus* of a probable bacterial-origin gene encoding an endo-1,4-beta-mannosidase enzyme (MAN4, EC 3.2.1.78). This likely ancient LGT event provides an interesting vignette that further illustrates gene structure evolution processes within the Hemiptera. Inspection of genome assemblies and predicted protein accessions reveals that this LGT event is shared with the stink bug *Halyomorpha halys*, a member of the same infraorder (Pentatomomorpha), but was introduced after this lineage diverged from other hemipterans, including the bed bug (Fig. 8a). Furthermore, whereas *Oncopeltus* now has two copies of this gene, independently the original *Halyomorpha* gene underwent a series of tandem duplications leading to nine extant copies (Fig. 8b, see also Fig. S2.6). Since the original LGT event, the *mannosidase* genes in both bug species have become

641 "domesticated" as multi-exonic genes (as in [78]). Moreover, as the splice site pattern
642 is unique to each species and evinces subsequent splice introductions in subsets of
643 paralogs (Fig. 8c), *mannosidase* genes further illustrate the hemipteran penchant for
644 intron introduction and maintenance of small exons. The retention and subsequent
645 expansion of these genes implies their positive selection, consistent with the
646 phytophagous diet of these hemipteran species. In this context, it is tempting to
647 speculate further that the marked proliferation of this enzyme in the stink bug
648 correlates with the breadth of its diet, as this agricultural pest feeds on a number of
649 different tissues in a range of host plants [79].
650
651
652 **Cuticle development, structure, and warning pigmentation**
653 Given the milkweed bug's history as a powerful model for endocrine studies of
654 hemimetabolous molting and metamorphosis since the late 1960's [21, 80-83], we
655 next focused on genes underlying the development and structural properties of the
656 *Oncopeltus* cuticle. Molting is triggered by the release of ecdysteroids, steroid
657 hormones that are synthesized from cholesterol in the prothoracic gland by
658 cytochrome P450 enzymes of the Halloween family [84], and we were able to identify
659 these in the *Oncopeltus* genome (see also Supplemental Notes 5.2.b, 5.3.b for these
660 and following metamorphosis gene details). From the ecdysone response cascade
661 defined in *Drosophila* [85], we identified *Oncopeltus* orthologs of both early and late-
662 acting factors. It will be interesting to see if the same regulatory relationships are
663 conserved in the context of hemimetabolous molting in *Oncopeltus*. For example,
664 *E75A* is required for reactivation of ecdysteroid production during the molt cycle in
665 *Drosophila* larvae [86] and likely operates similarly in *Oncopeltus*, since *Of-E75A*
666 RNAi prevents fourth-instar nymphs from molting to the fifth instar (H. Kelstrup and
667 L. Riddiford, unpublished data). In holometabolous insects, a declining titer of
668 ecdysteroids leads to the release of a series of neuropeptides that ultimately causes the
669 insect to molt, or ecdyse [87, 88]. Orthologs of these hormones and their receptors
670 are also present in the *Oncopeltus* genome or transcriptomic data.
671
672 In hemipterans, activation of juvenile hormone (JH) signaling at molts
673 determines whether the insect progresses to another nymphal instar or, if lacking,
674 becomes an adult [47]. We were able to identify many components of the JH signal
675 transduction pathway in the *Oncopeltus* genome, including an ortholog of
676 *Methoprene-tolerant* (*Met*), the JH receptor [47, 89], and the JH-response gene *Kr-h1*
677 [47, 90, 91]. JH acts to determine cuticle identity through regulation of the *broad*
678 gene in a wide variety of insects, where different isoforms direct specific aspects of
679 metamorphosis in *Drosophila* [92, 93]. In *Oncopeltus*, *broad* expression directs
680 progression through each of the nymphal stages [94], but the effect of each isoform
681 was unknown. We identified three isoforms in *Oncopeltus* – Z2, Z3, and Z4 – and
682 performed isoform-specific RNAi. In contrast to *Drosophila*, Broad isoform
683 functions appear to be more redundant in *Oncopeltus*, as knockdown of isoforms Z2
684 and Z3 have similar effects on survival to adulthood as well as adult wing size and
685 morphology (Fig. 9).
686
687 Regulators such as Broad initiate the transcription of a large battery of genes
688 that encode the structural components of the cuticle needed at each molt, consistent
689 with our expression analyses (Fig. 2b, discussed above). We identified 173 genes
690 encoding putative cuticle structural proteins in the milkweed bug, using established

sequence motifs (see also Supplemental Note 5.2.c). Similar to other insects, the CPR family, with the RR-1 (soft cuticle), RR-2 (hard cuticle), and unclassifiable types, constituted the largest of the cuticle protein gene groups. While several protein families are similar in size to those of other insects (CPAP1, CPAP3, and TWDL: see also Table S5.12), we found a slight expansion in the *Oncopeltus* CPF family (see also Fig. S5.14). For cuticle production, similar to the bed bug and the Asian longhorned beetle [11, 29], we identified a single *chitin synthase* gene with conserved alternative splice isoforms, which suggests that *chitin synthase 2* is a duplication specific to only certain beetle and fly lineages within the Holometabola [95].

One of the major characteristics of the milkweed bug is the distinctive red-orange and black aposematic (warning) coloration within the cuticle and epidermis that has been shown to act as a deterrent to predators (*e.g.*, Figs. 1, 9, [19, 20]). For black coloration, we were able to identify the key melanin synthesis enzymes (see also Fig. S5.15). The melanin synthesis pathway is conserved across holometabolous insects (*e.g.*, [96, 97]), and recent work in *Oncopeltus* [98, 99] supports functional conservation of melanin production in hemimetabolous lineages as well. In contrast, production of the primary warning coloration produced by the pteridine red erythropterin [100] remains an open avenue for hemimetabolous research. This pigment, along with other pterins, is synthesized from GTP through a series of enzymatic reactions [101]. The genes encoding enzymes that convert GTP into pterins have not been as extensively studied as melanins, and thus far in *Oncopeltus* we were only able to identify orthologs of *punch*, which encodes a GTP cyclohydrolase [102], and *sepia*, which is required for the synthesis of the red eye pigment drosopterin [103]. The bright red color of *Oncopeltus* eggs may in part reflect chemical protection transmitted parentally [104]. Thus, further identification of pigmentation genes will provide fitness indicators for maternal contributions to developmental success under natural conditions (*i.e.*, the presence of egg predators).

Chemoreception and metabolism in relation to feeding biology

The aposematic pigmentation of the milkweed bug advertises the fact that toxins in its milkweed seed diet are incorporated into the bugs themselves, a metabolic feat that was independently acquired in *Oncopeltus* and in the similarly colored monarch butterfly (*Danaus plexippus*), which shares this food source [35, 105]. Moreover, given the fundamental differences in metabolic pathways between phytophagous, mucivorous, and hematophagous species, we investigated to what extent differences in feeding ecology across hemipterans are represented in the chemoreceptor and metabolic enzyme repertoires of these species.

The ability of insects to smell and taste the enormous diversity of chemicals important to them for locating and identifying food, mates, oviposition sites, and other aspects of their environment is primarily mediated by three large gene families. The closely related Odorant Receptor (OR) and Gustatory Receptor (GR) families, and the distinct Ionotropic Receptor (IR) family [106-109], commonly encode tens to hundreds of chemoreceptors in arthropods. Consistent with having a less derived feeding ecology than species with phloem-restricted or obligate hematophagous diets, *Oncopeltus* retains a moderate complement of chemoreceptors from the different classes (Table 2, see also Supplemental Note 5.3.f). The hematophagous *Cimex* and *Rhodnius* have relatively depauperate OR and GR families compared to *Oncopeltus*.

While a few conserved orthologs such as the OrCo protein and a fructose receptor are found across species, *Oncopeltus* and *Acyrthosiphon* retain a set of sugar receptors, a gene lineage lost independently from the blood-feeding bugs (*Rhodnius* [10], *Cimex* [11]) and body louse (*Pediculus* [110]). Conversely, *Oncopeltus* has, like *Cimex*, a set of candidate carbon dioxide receptors, a gene lineage lost from *Rhodnius*, *Acyrthosiphon*, and *Pediculus* [10, 11, 111], but which is similar to a GR subfamily expansion in the more distantly related hemimetabolous termite (Isoptera, [112]). Comparable numbers of IRs occur across the heteropterans, where in addition to a conserved set of orthologs primarily involved in sensing temperature and certain acids and amines, *Oncopeltus* has a minor expansion of IRs distantly related to those involved in taste in *Drosophila*. The major expansions in each insect lineage are the candidate "bitter" GRs ([113], see also Supplemental Note 5.3.f, Fig. S5.19). In summary, *Oncopeltus* exhibits moderate expansion of specific subfamilies likely to be involved in host plant recognition, consistent with it being a preferentially specialist feeder with a potentially patchy food source [20, 114].

Table 2. Numbers of chemoreceptor genes/proteins per family in selected insect species. In some cases the number of proteins is higher than the number of genes due to an unusual form of alternative splicing, which is particularly notable for the *Oncopeltus* GRs. Data are shown for four Hemiptera as well as *Drosophila melanogaster*, the body louse *Pediculus humanus*, and the termite *Zootermopsis nevadensis* [10, 11, 108, 110-112, 115].

Species	Odorant	Gustatory	Ionotropic
Oncopeltus fasciatus [1]	120/121	115/169	37/37
Cimex lectularius [1,2]	48/49	24/36	30/30
Rhodnius prolixus [1,2]	116/116	28/30	33/33
Acyrthosiphon pisum [3]	79/79	77/77	19/19
Pediculus humanus [2]	12/13	6/8	14/14
Zootermopsis nevadensis	70/70	87/90	150/150
Drosophila melanogaster	60/62	60/68	65/65

[1] Hemiptera: Heteroptera
[2] independent acquisitions of hematophagy [16]
[3] Hemiptera, phloem-feeding

As host plant recognition is only the first step, we further explored whether novel features of the *Oncopeltus* gene set may be directly associated with its diet. We therefore used the CycADS annotation pipeline [116] to reconstruct the *Oncopeltus* metabolic network. The resulting BioCyc metabolism database for *Oncopeltus* ("OncfaCyc") was then compared with those for 26 other insect species in the current ArthropodaCyc collection ([117], http://arthropodacyc.cycadsys.org/), including three other hemipterans: the pea aphid, the green peach aphid, and the kissing bug (Tables 3-4). For a global metabolism analysis, we detected the presence of 1085 Enzyme Commission (EC) annotated reactions with at least one protein in the *Oncopeltus* genome (see also Supplemental Note 6.4, Table S6.9). Among these, 10 enzyme

783 classes (represented by 17 genes) are unique and 17 are missing when compared to
784 the other insects (Table 4, Table S6.10).
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787
788 **Table 3. Hemipteran ArthropodaCyc database summaries.**
789 Overview statistics for the newly created database for *Oncopeltus fasciatus* (Ofas) in
790 comparison with public databases for *Rhodnius prolixus* (Rpro), *Acyrthosiphon pisum*
791 (Apis), and *Myzus persicae* (Mper) available from [117]. Based on OGS v1.1.
792

Species ID Gene set ID	*Ofas* OGS v1.1	*Rpro* RproC1.1 (Built on RproC1 assembly)	*Apis* OGS v2.1b (Built on Acyr_2.0 assembly)	*Mper* Clone G006 v1.0	*Mper* Clone O v1.0
CycADS Database ID	OncfaCyc	RhoprCyc	AcypiCyc v2.1b	Myzpe_G006 Cyc	Myzpe_O Cyc
Total mRNA[1]	19,673	15,437	36,195	24,814	24,770
Pathways	294	312	307	319	306
Enzymatic Reactions	2,192	2,366	2,339	2,384	2,354
Polypeptides	19,820	15,471	36,228	24,849	24,805
Enzymes	3,050	2,660	5,087	4,646	4,453
Compounds	1,506	1,665	1,637	1,603	1,655

793
794 [1] In the BioCyc databases all splice variants are counted in the summary tables for
795 genes.
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799
800 **Table 4. Hemipteran ArthropodaCyc annotations of metabolic genes.**
801 Taxonomic abbreviations are as in Table 3.
802

	Ofas	*Rpro*	*Apis*	*Mper*
		Global metabolism		
EC[1] present in the genome	1085	1241	1288	1222
EC unique to this genome[2]	10	13	23	5
EC missing only in this genome[2]	17[4]	8	2	6
		Amino acid metabolism (KEGG)		
EC present in the genome	169	188	195	185
EC unique to this genome[2]	2	1	6	1
EC missing only in this genome[2]	5	2	0	2
EC unique to this genome[3]	8	10	12	8
EC missing only in this genome[3]	14	5	0	2

803
804 [1] "EC" refers to the number of proteins, as represented by their unique numerical
805 designations within the Enzyme Commission (EC) classification system for enzymes
806 and their catalytic reactions.
807 [2] in comparison to all other insects from ArthropodaCyc
808 [3] in comparison among the four hemipterans
809 [4] includes three EC categories added in OGS v1.2 (see also Table S6.10)
810

We then looked specifically at amino acid metabolism in four hemipterans representing the three different diets (see also Table S6.11). Among eight EC annotated enzymes present in the milkweed bug genome but not the other three, we identified the arginase (E.C. 3.5.3.1) that degrades arginine (Arg) into urea and ornithine, a precursor of proline (Pro). Given this difference, we extended our analysis to the entire urea cycle (Fig. 10a). Across all 26 insects present in the database, we identified three distinct groups (see also Table S6.12): (i) *Oncopeltus* and six other non-hemipteran insects that are able to degrade Arg but cannot synthesize it (Fig. 10b); (ii) the other three hemipterans that uniquely can neither synthesize nor degrade Arg via this cycle (Fig. 10c); and (iii) the other 17 insects that, with some minor differences, have an almost complete cycle (Fig. 10d). This suggests that loss of the ability to synthesize Arg may already have occurred at the base of the Hemiptera, with subsequent, independent loss of Arg degradation capacity in the aphid and *Rhodnius* lineages. Retention of Arg degradation in *Oncopeltus* might be linked to the milkweed seed food source, as most seeds are very rich in Arg [118], and Arg is indeed among the metabolites detected in *Oncopeltus* [119]. However, the monarch butterfly is one of only a handful of species that retains the complete Arg pathway (Fig. 10d: blue text). Despite a shared food source, these species may therefore differ in their overall Arg requirements, or – in light of a possible group benefit of *Oncopeltus* aggregation during feeding [20] – in their efficiency of Arg uptake.

Other enzymes are also present only in the milkweed bug in comparison with the other hemipterans (see also Table S6.11). As would be expected, *Oncopeltus*, like other insects [117], has the ability to degrade tyrosine (Tyr), a pathway that was uniquely lost in the aphids. Given the variable yields of Tyr from a mucivorous diet [120], this amino acid needed for cuticle maturation (sclerotization) is jointly synthesized – and consumed – by the aphid host and its endosymbiotic bacteria [5, 6, 15, 121]. Meanwhile, we find support for the recent nature of milkweed bug lineage-specific duplications that led to three copies of the Na+/K+ ATPase alpha subunits whose amino acid substitutions confer increased resistance to milkweed cardenolides [35, 122]. In the *Oncopeltus* genome, the genes encoding subunits ATPα1B and ATPα1C occur as a tandem duplication, notably on a scaffold that also harbors one of the clustered ZF271-like gene expansions (see above).

CONCLUSIONS

The integrated genomic and transcriptomic resources presented here for the milkweed bug *Oncopeltus fasciatus* (Figs. 2,5) underpin new insights into molecular evolution and suites of related biological characters within the Hemiptera. The gene structure trends we identified, with lineage predominating over genome size as a predictor and with many intron gains in the hemipteroid lineage (Figs. 6,7), offer initial parameters and hypotheses for the Hemiptera, Coleoptera, and Diptera. Such ordinal-level parameters can be evaluated against new species' data and also inform customized pipelines for future automated gene model predictions. At the same time, it will be interesting to explore the ramifications of hemipteroid intron gains. For example, while possessing more, small exons brings an increased transcriptional cost, it may

also provide greater scope to generate protein modularity via isoforms based on alternative exon usage. Furthermore, with the larger genome sizes and lower gene densities of hemipteroids compare to the well-studied Hymenoptera, it will also be interesting to see whether and in which direction hemipteroid gene and intron size may correlate with recombination rates [123].

Our analyses also highlight new directions for future experimental research, building on *Oncopeltus*'s long-standing history as a laboratory model and its active research community in the modern molecular genetics era (*e.g.*, Fig. 9, [24-26]). Functional testing will clarify the roles of genes we have identified as unique to the Hemiptera, including those implicated in chemical protection, bacterial and plant cell wall metabolism, or encoding wholly novel proteins (Figs. 3,8, see also Supplemental Note 2.2). Meanwhile, the prominent and species-specific expansions specifically of ZF271-like zinc fingers (Fig. 4), combined with the absence of the co-repressor KAP-1 in insects, argues for investigation into alternative possible interaction partners, which could clarify the nature of these zinc fingers' regulatory role and their binding targets.

One key output of this study is the generation of a metabolism database for *Oncopeltus*, contributing to the ArthropodaCyc collection (Table 3). In addition to comparisons with other species (Fig. 10), this database can also serve as a future reference for studies that use *Oncopeltus* as an ecotoxicology model species (*e.g.*, [124]). While we have primarily focused on feeding ecology in terms of broad comparisons between phytophagy and fluid feeding, *Oncopeltus* is also poised to support future work on nuances among phytophagous species. Despite its milkweed diet in the wild, the lab strain of *Oncopeltus* has long been adapted to feed on sunflower seeds, demonstrating a latent capacity for more generalist phytophagy [114]. This potential may also be reflected in a larger gustatory receptor repertoire than would be expected for an obligate specialist feeder (Table 2). Thus, *Oncopeltus* can serve as a reference species for promiscuously phytophagous pest species such as the stink bug. Finally, given that we have identified a number of key genes implicated in life history trade-offs, the genome data represent an important tool to explore the proximate mechanisms of fundamental aspects of life history evolution in an organism in which the ultimate explanations for traits such as cardenolide tolerance, pigmentation, and plasticity in reproduction under environmental variation have been elucidated in both the laboratory and nature.

METHODS

(More information is available in the supplementary materials, Additional file 1.)

Milkweed bug strain, rearing, and DNA/RNA extraction

The milkweed bug *Oncopeltus fasciatus* (Dallas), Carolina Biological Supply strain (Burlington, North Carolina, USA), was maintained in a laboratory colony under standard husbandry conditions (sunflower seed and water diet, 25 ºC, 12:12 light-dark photoperiod). Voucher specimens for an adult female (record # ZFMK-TIS-26324) and adult male (record # ZFMK-TIS-26325) have been preserved in ethanol and deposited in the Biobank of the Centre for Molecular Biodiversity Research, Zoological Research Museum Alexander Koenig, Bonn, Germany (https://www.zfmk.de/en/biobank).

Genomic DNA was isolated from individual, dissected adults using the Blood & Cell Culture DNA Midi Kit (G/100) (Qiagen Inc., Valencia, California, USA). Total RNA was isolated from individual, dissected adults and from pooled, mixed-instar nymphs with TRIzol Reagent (Invitrogen/ Thermo Fisher Scientific, Waltham, Massachusetts, USA). Dissection improved accessibility of muscle tissue by disrupting the exoskeleton, and gut material was removed.

Genome size calculations (flow cytometry, *k*-mer estimation)

Genome size estimations were obtained by flow cytometry with Hare and Johnston's protocol [125]. Four to five females and males each from the Carolina Biological Supply lab strain and a wild strain (collected from Athens, Georgia, USA; GPS coordinates: 33° 56' 52.8216" N, 83° 22' 38.3484'' W) were measured (see also Supplemental Note 2.1.a). At the bioinformatic level, we attempted to estimate genome size by *k*-mer spectrum distribution analysis for a range of *k*=15 to 34 counted with Jellyfish 2.1.4 [126] and bbmap [127], graphing these counts against the frequency of occurrence of *k*-mers (depth), and calculating genome size based on the coverage at the peak of the distribution (see also Supplemental Note 2.1.b).

Genome sequencing, assembly, annotation, and official gene set overview

Library preparation, sequencing, assembly, and automatic gene annotation were conducted at the Baylor College of Medicine Human Genome Sequencing Center (as in [11, 29]). About 1.1 billion 100-bp paired-end reads generated on an Illumina HiSeq2000s machine were assembled using ALLPATHS-LG [128], from two paired-end (PE) and two mate pair (MP) libraries specifically designed for this algorithm (see also Supplemental Note 1). Three libraries were sequenced from an individual adult male (180- and 500-bp PE, 3-kb MP), with the fourth from an individual adult female (8-10-kb MP). The final assembly (see metrics in Table 1) has been deposited in GenBank (accession GCA_000696205.1).

Automated annotation of protein-coding genes was performed using a Maker 2.0 annotation pipeline [129] tuned specifically for arthropods (see also Supplemental Note 3). These gene predictions were used as the starting point for manual curation via the Apollo v.1.0.4 web browser interface [130], and automatic and manual curations were compiled to generate the OGS (see also Supplemental Note 4). Databases of the genome assembly, Maker automatic gene predictions, and OGS v1.1 are available through the i5K Workspace@NAL [131], and the Ag Data Commons data access system of the United States Department of Agriculture's (USDA) National

Agricultural Library as individual citable databases [132-134]. The current version of the gene set, OGS v1.2, will be deposited in NCBI under accession number XXX.

Repeat content analysis

Repetitive regions were identified in the *Oncopeltus* genome assembly with RepeatModeler Open-1.0.8 [135] based on a species-specific repeat library generated *de novo* with RECON [136], RepeatScout [137], and Tandem Repeats Finder [138]. Then, RepeatMasker Open-4.0 [139] was used to mask repeat sequences based on the RepeatModeler library. Given the fragmented nature of the assembly, we attempted to fill and close assembly gaps by sequencing additional material, generating long reads with single molecule real time sequencing on a PacBio RS II machine (34 SMRT cells to an expected coverage of 8x, see also Supplemental Note 2.3). Gap filling on the Illumina assembly scaffolds was performed with PBJelly version 13.10.22, and the resulting assembly was used for repeat content estimation and comparison with *Cimex lectularius* and *Acyrthosiphon pisum.*

Transcriptome resources

Total RNA from three distinct life history samples (pooled, mixed-instar nymphs; an adult male; an adult female) was also sequenced on an Illumina HiSeq2000s machine, producing a total of 72 million 100-bp paired-end reads (see also Supplemental Note 1.3, Table S1.1). These expression data were used to support the generation of the OGS at different stages of the project: as input for the evidence-guided automated annotation with Maker 2.0 (see also Supplemental Note 3), as expression evidence tracks in the Apollo browser to support the community curation of the OGS, and, once assembled into a *de novo* transcriptome, as a point of comparison for quality control of the OGS.

The raw RNA-seq reads were pre-processed by filtering out low quality bases (phred score <30) and Truseq adapters with Trimmomatic-0.30. Further filtering removed ribosomal and mitochondrial RNA sequences with Bowtie 2 [140], based on a custom library built with all hemipteran ribosomal and mitochondrial RNA accessions from NCBI as of 7th February 2014 (6,069 accessions). The pooled, filtered reads were mapped to the genome assembly with Tophat2-PE on CyVerse [141]. A second set of RNA-seq reads from an earlier study ("published adult" dataset, [35]) was also filtered and mapped in the same fashion, and both datasets were loaded into the *Oncopeltus* Apollo instance as evidence tracks (under the track names "pooled RNA-seq - cleaned reads" and "RNA-seq raw PE reads Andolfatto et al", respectively).

Additionally, a *de novo* transcriptome was generated from our filtered RNA-seq reads (pooled from all three samples prepared in this study) using Trinity [142] and TransDecoder [143] with default parameters. This transcriptome is referred to as "i5K", to distinguish it from a previously published maternal and early embryonic transcriptome for *Oncopeltus* (referred to as "454", [34]). Both the i5K and 454 transcriptomes were mapped to the genome assembly with GMAP v. 2014-05-15 on CyVerse. These datasets were also loaded into the Apollo browser as evidence tracks to assist in manual curation.

Life stage specific expression analyses

Transcript expression of the OGS v1.1 genes was estimated by running RSEM2 [144] on the filtered RNA-seq datasets for the three postembryonic stages against the OGS v1.1 cDNA dataset. Transcript expression was then based on the transcripts per million (TPM) value. The TPM values were processed by adding a value of 1 (to avoid zeros) and then performing a log2-transformation. The number of expressed genes per RNA-seq library was compared for TPM cutoffs of >1, >0.5, and >0.25. For this first-pass expression assessment, a >0.25 cutoff was chosen, which reduced the number of expressed genes by 6.6% compared to the first analysis, while the other TPM cutoffs were deemed too restrictive (reducing the expressed gene set by 10.3% and 16.6%, respectively, with the >0.5 and >1 cutoffs). This analysis was also applied to the "published adult" dataset [35]. To include embryonic stages in the comparison, transcripts from the 454 transcriptome were used as blastn queries against the OGS v1.1 cDNA dataset (cutoff e-value $<10^{-5}$). The results from all datasets were converted to binary format to generate Venn diagrams (Fig. 2b).

Protein gene orthology assessments via OrthoDB and BUSCO analyses

These analyses follow previously described approaches [41, 42]. See Supplemental Note 6.1 for further details.

Global transcription factor identification

Likely transcription factors (TFs) were identified by scanning the amino acid sequences of predicted protein-coding genes for putative DNA binding domains (DBDs), and when possible, the DNA binding specificity of each TF was predicted using established procedures [56]. Briefly, all protein sequences were scanned for putative DBDs using the 81 Pfam [145] models listed in Weirauch and Hughes [146] and the HMMER tool [147], with the recommended detection thresholds of Per-sequence Eval < 0.01 and Per-domain conditional Eval < 0.01. Each protein was classified into a family based on its DBDs and their order in the protein sequence (e.g., bZIPx1, AP2x2, Homeodomain+Pou). The resulting DBD amino acid sequences were then aligned within each family using Clustal Omega [148], with default settings. For protein pairs with multiple DBDs, each DBD was aligned separately. From these alignments, the sequence identity was calculated for all DBD sequence pairs (*i.e.*, the percent of amino acid residues that are identical across all positions in the alignment). Using previously established sequence identity thresholds for each family [56], the predicted DNA binding specificities were mapped by simple transfer. For example, the DBD of OFAS001246-RA is 98% identical to the *Drosophila melanogaster* Bric a Brac 1 (Bab1) protein. Since the DNA binding specificity of Bab1 has already been experimentally determined, and the cutoff for the Pipsqueak family TFs is 85%, we can infer that OFAS001246-RA will have the same binding specificity as *Drosophila* Bab1.

RNA interference

Double-stranded RNA (dsRNA) was designed to target the final, unique exon of the *broad* isoforms *Z2, Z3*, and *Z4*. A portion of the coding sequence for the zinc finger region from these exons (179 bp, 206 bp, and 216 bp, respectively) was cloned into a plasmid vector and used as template for *in vitro* RNA synthesis, using the gene-specific primer pairs: Of-Z2_fwd: 5′-ATGTGGCAGACAAGCATGCT-3′; Of-Z2_rev: 5′-CTAAAATTTGACATCAGTAGGC-3′; Of-Z3_fwd: 5′-ccttctcctgttactactcac-3′; Of-Z3_rev: 5′-ttatatgggcggctgtccaa-3′; Of-Z4_fwd: 5′-

AACACTGACCTTGGTTACACA-3′; Of-Z4_rev: 5′-TAGGTGGAGGATTGCTAAAATT-3′. Two separate transcription reactions (one for each strand) were performed using the Ambion MEGAscript kit (Ambion, Austin, Texas, USA). The reactions were purified by phenol/chloroform extraction followed by precipitation as described in the MEGAscript protocol. The separate strands were re-annealed in a thermocycler as described previously [31]. Nymphs were injected with a Hamilton syringe fitted with a 32-gauge needle as described [53]. The concentration of *Of-Z2*, *Of-Z3* and *Of-Z4* dsRNA was 740 ng/µl, 1400 ng/µl, and 1200 ng/µl, respectively. All nymphs were injected within 8 hours of the molt to the fourth (penultimate juvenile) instar (n ≥12 per treatment: see Fig. 9). Fore- and hindwings were then dissected from adults and photographed at the same scale as wings from wild type, uninjected controls.

CycADS annotation and OncfaCyc database generation

We used the Cyc Annotation Database System (CycADS, [116]), an automated annotation management system, to integrate protein annotations from different sources into a Cyc metabolic networks reconstruction that was integrated into the ArthropodaCyc database. Using our CycADS pipeline, *Oncopeltus fasciatus* proteins from the official gene set OGS v1.1 were annotated using different methods – including KAAS [149], PRIAM [150], Blast2GO [151, 152], and InterProScan with several approaches [153] – to obtain EC and GO numbers. All annotation information data were collected in the CycADS SQL database and automatically extracted to generate appropriate input files to build or update BioCyc databases [154] using the Pathway Tools software [155]. The OncfaCyc database, representing the metabolic protein-coding genes of *Oncopeltus*, was thus generated and is now included in the ArthropodaCyc database, a collection of arthropod metabolic network databases ([117], http://arthropodacyc.cycadsys.org/).

FIGURE LEGENDS

Fig. 1. The large milkweed bug, *Oncopeltus fasciatus*, shown in its phylogenetic and environmental context.

(a) Species tree of selected Hemiptera with genomic and transcriptomic resources, based on phylogenetic analyses and divergence time estimates in [3]. Species marked with an asterisk (*) have published resources; those with the appellation “i5K” are part of a current pilot project supported by the Baylor College of Medicine Human Genome Sequencing Center and the National Agricultural Library of the USDA. Note that recent analyses suggest the traditional infraorder Cimicomorpha, to which *Rhodnius* and *Cimex* belong, may be paraphyletic [16].
(b-c) Milkweed bugs on their native food source, the milkweed plant: gregarious nymphs of different instars on a milkweed seed pod (b), and pale, recently eclosed adults and their shed exuvia (c). Images were taken at Avalon Park and Preserve, Stony Brook, New York, USA, courtesy of Deniz Erezyilmaz, used with permission.
(d) Individual bugs, shown from left to right: first instar nymphs (ventral and dorsal views) and adults (dorsal and lateral views); images courtesy of Kristen Panfilio (nymphs) and Jena Johnson (adults), used with permission. The arrow labels the labium (the “straw”), part of the hemipteran mouthpart anatomy adapted for feeding by piercing and sucking.

Fig. 2. Comparisons of the official gene set and transcriptomic resources.
(a) Area-proportional Venn diagram comparing the OGS v1.1 ("OGS"), a Trinity *de novo* transcriptome from the three post-embryonic RNA-seq samples ("i5K"), and the maternal and embryonic transcriptome from 454 data ("454" [34]). Sample sizes and the fraction of each transcriptome represented in the OGS are indicated (for the 454 dataset, only transcripts with homology identification were considered). The unique fraction of each set is also specified (%). Dataset overlaps were determined by blastn (best hit only, e-value $<10^{-9}$).
(b) Four-set Venn diagram representation of OGS v1.1 gene model expression across four different life history samples. Values are counts of gene models, with percentages also given for the largest subsets. Note that the "Embryo/Maternal" sample derives from 454 pyrosequencing data and therefore has a smaller data volume than the other samples, which were generated with Illumina sequencing.

Fig. 3. Orthology comparisons and phylogenetic placement of *Oncopeltus fasciatus* among other Arthropoda.
(a) Comparisons of protein-coding gene content in 12 arthropod species, with the Hemiptera highlighted in red text. The bar chart shows the number of proteins per conservation level (see legend), based on OrthoDB orthology clustering analyses. To the left is a maximum likelihood phylogeny based on concatenation of 395 single-copy orthologs (all nodes have 100% support unless otherwise noted; branch length unit is substitutions per site). The inset pie chart shows the proportion of proteins per conservation level in *Oncopeltus* (*"Ofas"*). See also Supplemental Note 6.1.
(b) Proportion of *Oncopeltus* proteins that have expression and/or curation validation support per conservation level (same color legend as in (a)). Expression support is based on the life history stage data in Fig. 2b.
(c) Protein orthology data evaluated by taxonomic grouping, based on the OrthoDB v8 i5K "Insecta" analysis with 64 species, a subset of the recently released OrthoDB v9 (http://www.orthodb.org): Hemiptera (red, 8 species); other hemimetabolous species (paraphyletic, yellow, 6 species); Holometabola (purple, 50 species). Values are given for both orthogroups (black text, defined in [42]) and protein-coding genes (blue text). As analyses that require all species to be represented in a given orthogroup are limited by the quality of every species' OGS, the cutoff for orthogroup presence in a given Venn diagram set was rather that roughly half of all relevant species are included, and strictly no species from a different set are permitted. Set [A] contains ≥10 hemimetabolous species (allowing for 4-8 Hemiptera and 2-6 other hemimetabolous species). Set [B] contains ≥4 Hemiptera and ≥25 Holometabola. Set [C] contains ≥2 other hemimetabolous species and for the Holometabola at least one representative from each of the Hymenoptera, Coleoptera, Lepidoptera, and Diptera. Analyses based on OGS v1.1.

Fig. 4. Distribution of transcription factor families across insect genomes.
(a) Heatmap depicting the abundance of transcription factor (TF) families across 17 insect genomes (Hemiptera highlighted in red text), with *Daphnia* as an outgroup. Each entry indicates the number of TF genes for the given family in the given genome, based on the presence of predicted DNA binding domains (see Methods). The color key has a log (base 2) scale (light blue means the TF family is completely absent). Values are in Supplementary Table S6.2.
(b) Bar graph showing the number of proteins of each of the two most abundant TF families, homeodomains and C2H2 zinc fingers (ZFs), per species. Solid lines

demarcate insect orders: Hemiptera (Hemipt.), Hymenoptera (Hym.), Coleoptera (Col.), and Diptera (Dipt.). The dashed line demarcates the dipteran family Culicidae (mosquitoes).
(c) Proportions of *Oncopeltus* homeodomain (HD) and C2H2 zinc finger proteins with orthology assignment (predicted DNA binding specificity) and/or manual curation. "Classified" refers to automated classification of a protein to a TF family, but without a specific orthology assignment.
(d) Maximum likelihood phylogeny of representative subsets of the zinc finger 271-like family in *Oncopeltus* (49 proteins, blue text) and protein accessions retrieved from GenBank for the pea aphid (55 proteins, black text) as well as chelicerate (red text) and holometabolan (yellow text) outgroups (16 proteins, 7 species). Gaps were removed during sequence alignment curation, with default pipeline settings [156]. All nodes have ≥50% support. Key nodes are circled for the distinct clades containing all aphid or all *Oncopeltus* proteins (82% support each), and for each 'core' clade comprised exclusively of proteins from each species (97% and 100%, respectively; triangles shown to scale for branch length and number of clade members). Branch length unit is substitutions per site.
Analyses based on OGS v1.1.

Fig. 5. Comparison of repeat content estimations.
(a) Comparison of total repetitive content among insect genomes. The three values for *Oncopeltus* are shown (in ascending order: original Illumina assembly, gap-filled assembly, Illumina-PacBio hybrid estimate). Values for the three hemipterans labeled in red text are from RepeatModeler (gold bars for the pea aphid and bed bug; blue and gold bars for *Oncopeltus*). All other values are from the respective genome papers, including a second value corresponding to the published repeat content for the first version of the aphid genome [5, 9, 112, 157-162]. Species abbreviations as in Fig. 4 (compare panels a and b), and additionally: *Nlug, Nilaparvata lugens; Lmig, Locusta migratoria; Bmor, Bombyx mori; Aalb, Aedes albopictus*.
(b) Comparison of repetitive element categories between three hemipteran genomes, based on results from RepeatModeler. Here we present assembly coverage as actual sequence length (Mb) to emphasize the greater repeat content in *Oncopeltus* (based on the gap-filled assembly: see also Supplemental Note 2.3).

Figure 6. Trends in gene structure show hemipteroid-specific tendencies.
(a) Trends in protein size, exon size, and exon number are shown for a highly conserved set of genes encoding large proteins of diverse functional classes ("gold standard", curated gene set). Median values are plotted. Sample sizes are indicated for each species, with 11 genes for which orthologs were evaluated in all species. Where it was not possible to analyze all 30 genes for a given species, equal sampling was done across the range of protein sizes of the complete dataset, based on the *Cimex* ortholog sizes (1:1:1 sampling from big:medium:small subcategories of 10 genes each). See also Supplemental Note 6.3.
(b) Box plot representations of coding sequence exon size (aa) for two species from each of three insect orders, based on datasets of unique coding sequence exons (one isoform per gene) and excluding terminal exons <10 aa (as most of those exons may rather be UTRs or a small placeholder N-terminal exon, as a byproduct of the Maker gene annotation pipeline's requirement to create nominally complete protein coding genes with in-frame start codons). Only manually curated gene models were considered for *Oncopeltus* and the other recent i5K species; the entire OGS was used

for *Tribolium* and *Drosophila*. For clarity, outliers are omitted; whiskers represent 1.5× the value of the Q3 (upper) or Q2 (lower) quartile range. MAD, median absolute deviation.
Species are represented by their four-letter taxonomic abbreviations, with their ordinal relationships given below the phylogeny in panel (a): Hemip., Hemiptera; Thys., Thysanoptera; Col., Coleoptera; Dipt., Diptera. Species abbreviations as in Fig. 4 and additionally: *Gbue, Gerris buenoi*; *Focc, Frankliniella occidentalis*; *Agla, Anoplophora glabripennis; Ccap, Ceratitis capitata*.

Fig 7. Splice site evolution correlates with both lineage and, independently, genome size.
Splice site changes are shown for *hemocytin* (blue text), *Tenascin major* (*Ten-m*, turquoise text), and *UDP-galactose 4′-epimerase* (brown text), mapped onto a species tree of eight insects. Patterns of splice site evolution were inferred based on the most parsimonious changes that could generate the given pattern within a protein sequence alignment of all orthologs (see also Supplemental Note 6.3 for complete methodology and data sources). In instances where an equal number of lineage specific gains or losses was possible, we remained agnostic and present a range for the ancestral number of splice sites indicated at the base of the tree, where the bracketed number indicates how many ancestral positions are still retained in all species. Along each lineage, subsequent changes are indicated in brackets, with the sign indicating gains (+) or losses (-). Values shown to the right are species-specific changes. Note that the values shown between the *D. melanogaster* and *T. castaneum* lineages denote changes that have occurred independently in both. Colored boxes highlight the largest sources of change, as indicated in the legend and discussed in the main text. Species are represented by their four-letter abbreviations (as defined in Fig. 6), and the estimated genome sizes are indicated parenthetically (measured size: [11, 29, 73, 161, 163]; draft assembly size: GenBank Genome IDs 14741 and 17730). Divergence times are shown in gray and given in millions of years [3]. Abbreviations as in Figs. 4,6, and: Col., Coleoptera; Dipt., Diptera; Hemip., Hemiptera; Hemipt., hemipteroid assemblage (including *F. occidentalis*); n.d., no data.

Fig. 8. Lateral gene transfer introduction and subsequent evolution within the Hemiptera for mannosidase-encoding genes.
(a) Species tree summary of evolutionary events. Stars represent the original LGT introduction and subsequent copy number gains (see legend).
(b) Maximum likelihood phylogeny of mannosidase proteins, including bacterial sequences identified among the best GenBank blastp hits for *Oncopeltus* and *Halyomorpha* (accession numbers as indicated, and for "Other bacteria" are: ACB22214.1, AEE17431.1, AEI12929.1, AEO43249.1, AFN74531.1, CDM56239.1, CUA67033.1, KOE98396.1, KPI24888.1, OAN41395.1, ODP26899.1, ODS11151.1, OON18663.1, PBD05534.1, SIR54690.1, WP096035621.1, YP001327394.1). All nodes have ≥50% support from 500 bootstrap replicates [164]. Triangles are shown to scale for branch length and number of clade members; branch length unit is substitutions per site.
(c) Manually curated protein sequence alignment for the N-terminal region, showing the position of splice sites ("|" symbol), where one position is ancestral and present in all paralogs of a given species (magenta), and one position occurs in a subset of paralogs and is presumed to be younger (cyan, note the position is within the 5′ UTR in the case of *Halyomorpha*). Residues highlighted in yellow are conserved between

the two species. The *Oncopeltus* paralog represented in the OGS as OFAS017153-RA is marked with an asterisk to indicate that this version of the gene model is incomplete and lacks the initial exon (gray text in the alignment). For clarity, only the final three digits of the *Halyomorpha* GenBank accessions are shown (full accessions: XP_014289XXX).

Fig. 9. Isoform-specific RNAi based on new genome annotations affects the molting and cuticle identity gene *broad*.
(a) Genomic organization of the cuticle identity gene *broad*. The regions used as template to generate isoform-specific dsRNA are indicated (red asterisks: the final, unique exons of each isoform). Previous RNAi studies targeted sequence within exons 1-5 that is shared among all isoforms (dashed red box, [94]).
(b) Knock down of the *Oncopeltus Z2* or *Z3 broad* isoforms at the onset of the penultimate instar resulted in altered nymphal survival and morphogenesis that was reflected in the size and proportion of the fore and hind wings at the adult stage (upper and lower images, respectively, shown to the same scale for all wings). We did not detect any effect on the wing phenotype when targeting the *Z4*-specific exon, demonstrating the specificity of the zinc finger coding region targeted by RNAi. Experimental statistics are provided in the figure inset, including for the buffer-injected negative control.

Fig. 10. Comparison of the urea cycle of *Oncopeltus* with 26 other insect species.
(a) Detailed diagram of the urea cycle (adapted from KEGG).
(b) Group of 7 species, including *Oncopeltus*, for which Arg degradation via arginase (3.5.3.1), but not synthesis, is possible.
(c) Group of 3 species for which neither the degradation nor synthesis of arginine via the urea cycle is possible (all 3 other hemipterans in this analysis).
(d) Group of 17 species sharing a complete (or almost complete) urea cycle.
Hemiptera are identified in red text and the milkweed-feeding monarch butterfly is in blue text. Enzyme names corresponding to EC numbers: 1.5.1.2 = pyrroline-5-carboxylate reductase; 1.14.13.39 = nitric-oxide synthase; 2.1.3.3 = ornithine carbamoyltransferase; 2.6.1.13 = ornithine aminotransferase; 3.5.3.1 = arginase; 4.3.2.1 = argininosuccinate lyase; 6.3.4.5 = argininosuccinate synthase.
Analyses based on OGS v1.1.

TABLES (see above within relevant manuscript sections)

Table 1. *Oncopeltus fasciatus* genome metrics.

Table 2. Numbers of chemoreceptor genes/proteins per family in selected insect species.

Table 3. Hemipteran ArthropodaCyc database summaries.

Table 4. Hemipteran ArthropodaCyc annotations of metabolic genes.

DECLARATIONS

ADDITIONAL FILES

Additional file 1: Supplementary figures, tables, methods, and other text. (PDF)
Additional file 2: Large supporting tables. (XLSX)
Additional file 3: Chemoreceptor sequences in FASTA format. (TXT)

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AVAILABILITY OF DATA AND MATERIALS

All sequence data are publically available at the NCBI, bioproject number PRJNA229125. In addition, gene models and a browser are available at the National Agricultural Library ([132-134], https://i5k.nal.usda.gov/Oncopeltus_fasciatus).

AUTHOR'S CONTRIBUTIONS

KAP and StR conceived, managed, and coordinated the project. KAP and SK provided specimens for sequencing and performed DNA and RNA extractions. StR, SD, SLL, HC, HVD, HD, YH, JQ, SCM, DSTH, KCW, DMM, and RAG constructed

libraries and performed sequencing. StR, SCM, and DSTH performed the genome assembly and automated gene prediction. IMVJ, JSJ, and PJM analyzed genome size. IMVJ, VK, PH, and KAP contributed to repetitive content analyses. AD, RR, JHW, KAP, and SK performed bacterial scaffold detection and LGT analyses. MCMT developed Apollo software. KAP, IMVJ, MCMT, CPC, C-YL, and MFP implemented Apollo-based manual curation. KAP, IMVJ, JBB, DE, YS, HMR, DA, CGCJ, BMIV, EJD, CSB, C-CC, Y-TC, ADC, AGC, AJJC, PKD, EMD, CGE, MF, NG, TH, Y-MH, ECJ, TEJ, JWJ, AK, ML, MRL, H-LL, YL, SRP, LP, MP, PNR, RRP, SiR, LS, MES, JS, ES, JNS, OT, LT, MVDZ, SV, and AJR participated in manual curation and contributed to the Supplemental Notes. IMVJ, KAP, DSTH, M-JMC, CPC, C-YL, and MFP performed curation quality control and generated the OGS. IMVJ, KAP, and JBB generated *de novo* transcriptomes and performed life history stage expression analyses. RMW, PI, KAP, and EMZ performed orthology and phylogenomic analyses. MTW, KAP, IMVJ, PH, and BMIV performed transcription factor analyses. EJD conducted analyses of DNA methylation. KAP, PH, and RJS contributed to comparative analyses of gene structure. DE conducted the RNAi experiments. SC, PB-P, GF, and NP generated and performed comparative analyses on the OncfaCyc database. KAP, IMVJ, JBB, DE, YS, SC, HMR, and MTW wrote the manuscript. KAP, IMVJ, JBB, DE, YS, SC, HMR, MFP, RMW, PI, MTW, StR, PJM, and AK edited the manuscript. IMVJ and KAP organized the Supplementary Materials. All authors approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

AUTHOR DETAILS

[1] Institute for Zoology: Developmental Biology, University of Cologne, Zülpicher Str. 47b, 50674 Cologne, Germany
[2] School of Life Sciences, University of Warwick, Gibbet Hill Campus, Coventry CV4 7AL, UK
[3] Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221, USA
[4] Department of Biochemistry and Cell Biology and Center for Developmental Genetics, Stony Brook University, Stony Brook, New York 11794, USA
[5] Department of Biological Sciences, Wellesley College, 106 Central St., Wellesley, Massachusetts 02481, USA
[6] Univ Lyon, INSA-Lyon, INRA, BF2I, UMR0203, F-69621, Villeurbanne, France
[7] Current address: LSTM, Laboratoire des Symbioses Tropicales et Méditerranéennes, INRA, IRD, CIRAD, SupAgro, University of Montpellier, Montpellier, France
[8] Department of Entomology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, USA
[9] National Agricultural Library, Beltsville, Maryland 20705, USA
[10] Department of Genetic Medicine and Development and Swiss Institute of Bioinformatics, University of Geneva, Geneva 1211, Switzerland
[11] Current address: Department of Ecology and Evolution, University of Lausanne, Lausanne 1015, Switzerland
[12] Center for Autoimmune Genomics and Etiology, Division of Biomedical Informatics, and Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45229, USA
[13] Human Genome Sequencing Center, Department of Human and Molecular Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA

[14] Current address: Department of Genome Sciences, University of Washington School of Medicine, Seattle, Washington 98195, USA
[15] Current address: Howard Hughes Medical Institute, University of Washington, Seattle, Washington 98195, USA
[16] Department of Biology, University of Rochester, Rochester, New York 14627, USA
[17] Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, Netherlands
[18] Max Planck Institute for Chemical Ecology, Hans-Knöll Strasse 8, 07745 Jena, Germany
[19] Department of Biochemistry and Genomics Aotearoa, University of Otago, Dunedin 9054, New Zealand
[20] School of Biology, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK
[21] Institut de Génomique Fonctionnelle de Lyon, Université de Lyon, Université Claude Bernard Lyon 1, CNRS UMR 5242, École Normale Supérieure de Lyon, 46 Allée d'Italie, 69364 Lyon, France
[22] Department of Ecology, Evolution and Behavior, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Edmond J. Safra Campus, Givat Ram 91904, Jerusalem, Israel
[23] Department of Entomology/Institute of Biotechnology, College of Bioresources and Agriculture, National Taiwan University, Taipei, Taiwan
[24] Current address: School of Life Sciences, Rochester Institute of Technology, Rochester, New York 14623, USA
[25] Department of Organismic and Evolutionary Biology, Harvard University, 26 Oxford Street, Cambridge, Massachusetts 02138, USA
[26] Department of Molecular and Cellular Biology, Harvard University, 26 Oxford Street, Cambridge, Massachusetts 02138, USA
[27] Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202, USA
[28] Institute for Genetics, University of Cologne, Zülpicher Straße 47a, 50674 Cologne, Germany
[29] Department of Entomology, Texas A&M University, College Station, Texas 77843, USA
[30] CECAD, University of Cologne, Cologne, Germany
[31] Department of Entomology and Program in Molecular & Cell Biology, University of Maryland, College Park, Maryland 20742, USA
[32] Department of Entomology, University of Georgia, 120 Cedar St., Athens, Georgia 30602, USA
[33] Environmental Genomics and Systems Biology Division, Lawrence Berkeley National Laboratory, Berkeley, California, USA
[34] Department of Entomology, College of Agriculture, Food and Environment, University of Kentucky, Lexington, Kentucky 40546, USA
[35] Department of Biology, University of Hawai'i at Mānoa, Honolulu, Hawaii 96822, USA
[36] Current address: Department of Evolutionary Genetics, Max-Planck-Institut für Evolutionsbiologie, August-Thienemann-Straße 2, 24306 Plön, Germany
[37] Current address: Earthworks Institute, 185 Caroline Street, Rochester, New York 14620, USA
[38] Centro de Bioinvestigaciones, Universidad Nacional del Noroeste de Buenos Aires, Argentina
[39] Current address: Department of Biotechnology, Central university of Rajasthan (CURAJ), NH-8, Bandarsindri, Ajmer- 305801, India
[40] Argelander-Institut für Astronomie, Universität Bonn, Auf dem Hügel 71, 53121 Bonn, Germany
[41] Current address: Department of Zoology, University of Cambridge, Cambridge CB2 3DT, UK
[42] Centro Regional de Estudios Genómicos, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina

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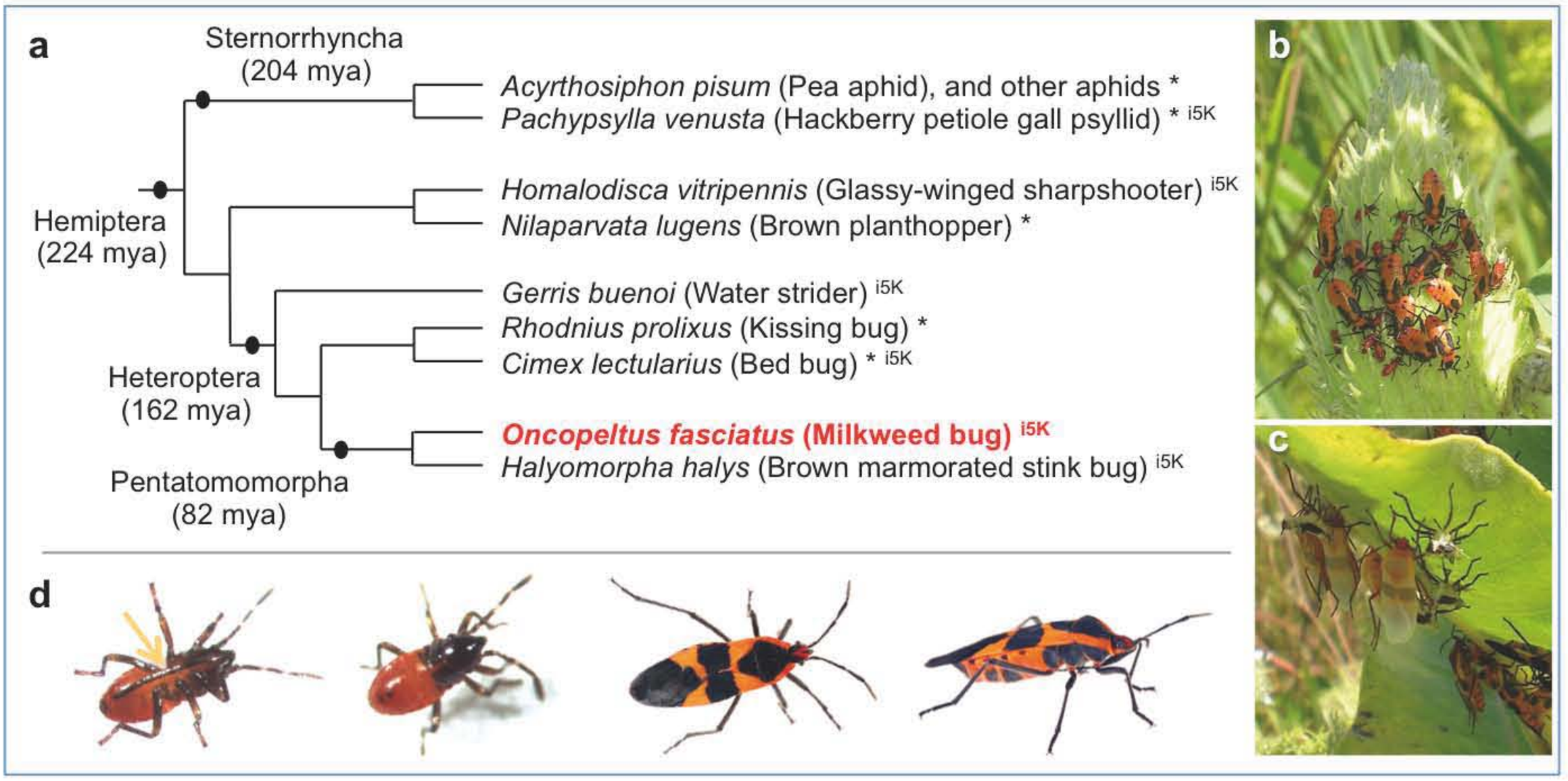


Fig 1. The large milkweed bug, *Oncopeltus fasciatus*, shown in its phylogenetic and environmental context.

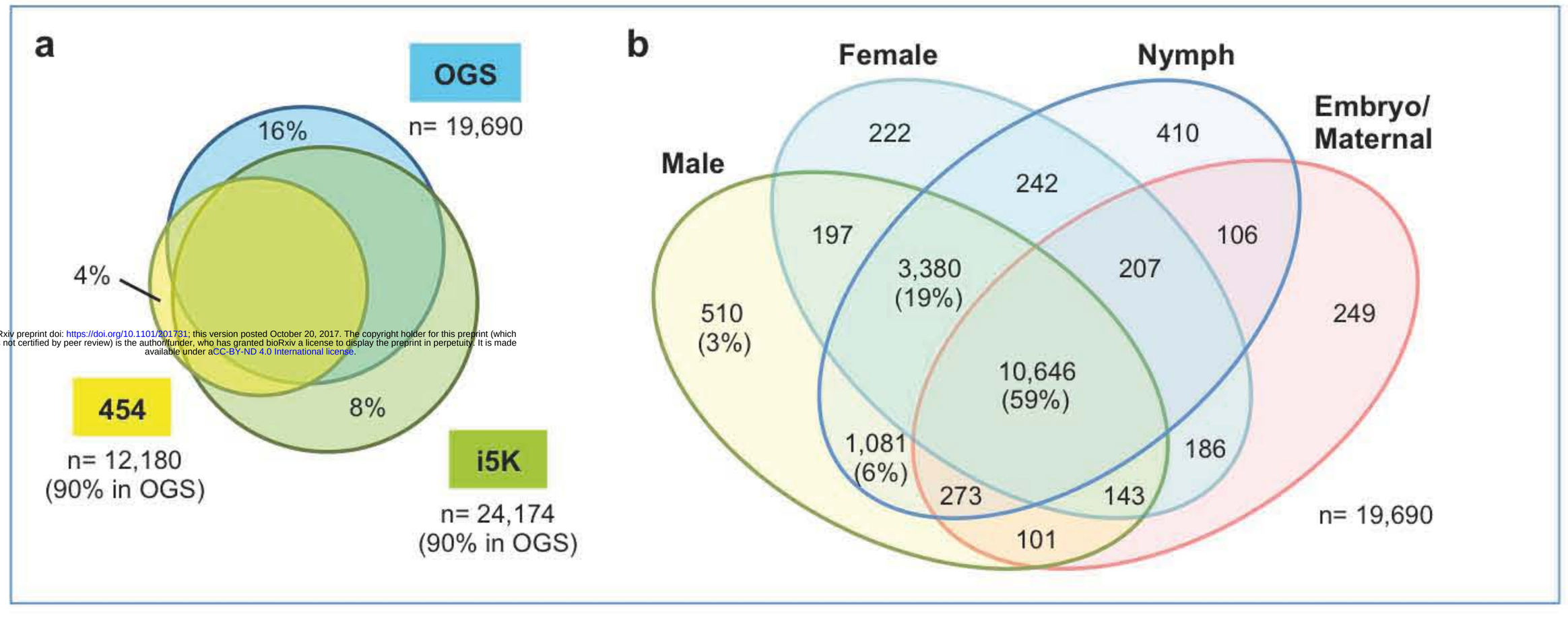


Fig 2. Comparisons of the official gene set and transcriptomic resources.

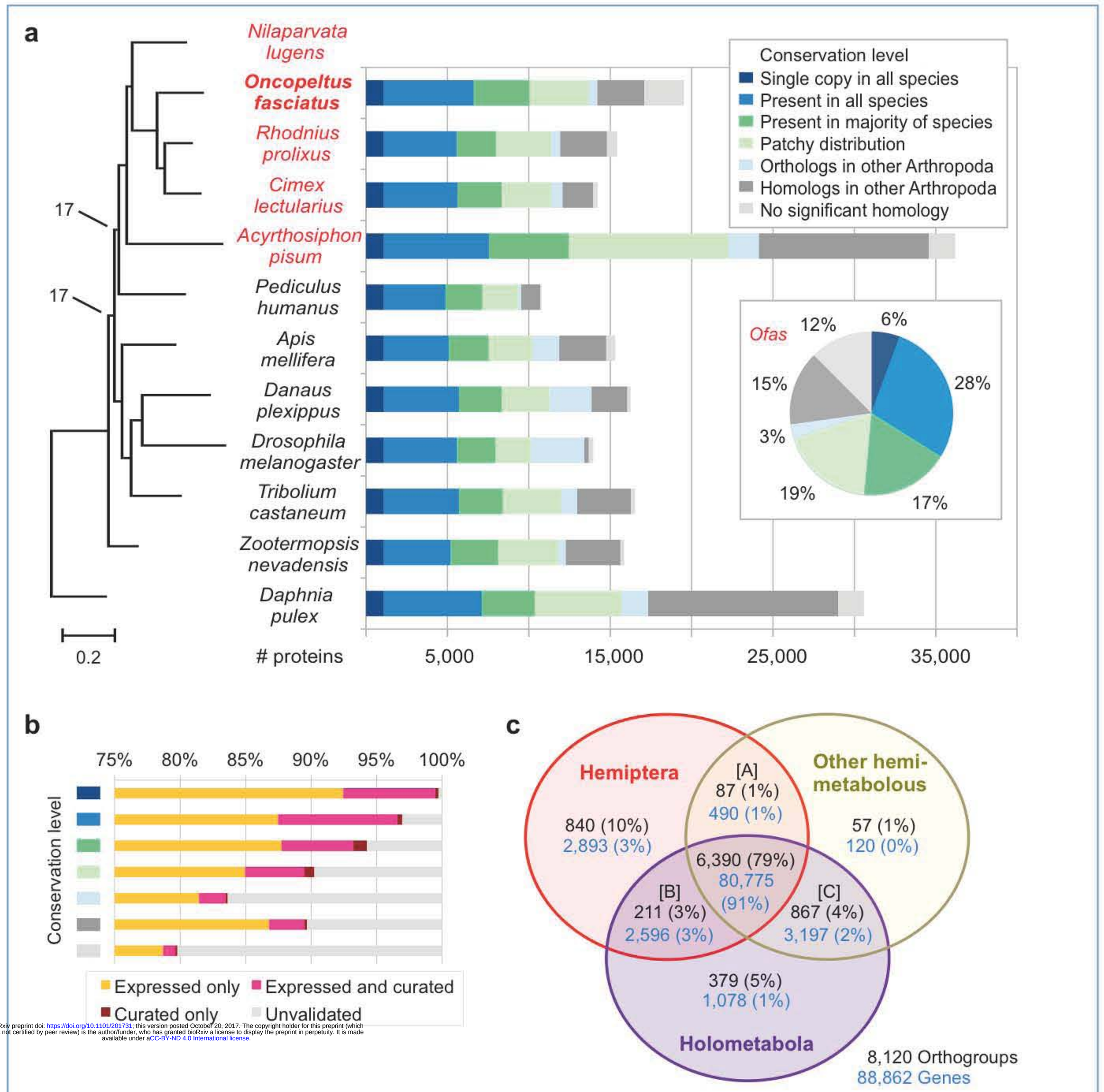


Fig 3. Orthology comparisons and phylogenetic placement of *Oncopeltus fasciatus* among other Arthropoda.

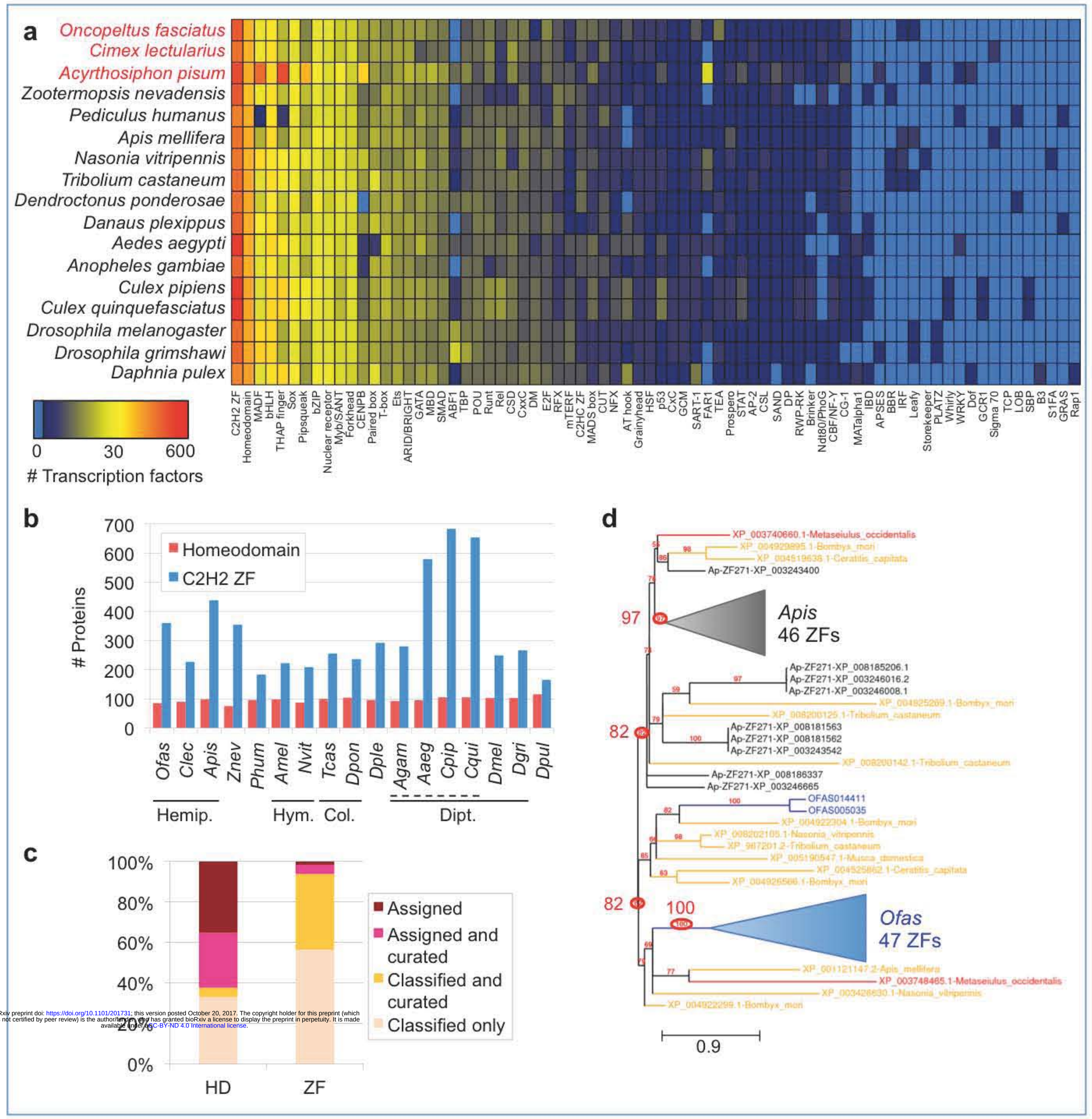


Fig 4. Distribution of transcription factor families across insect genomes.

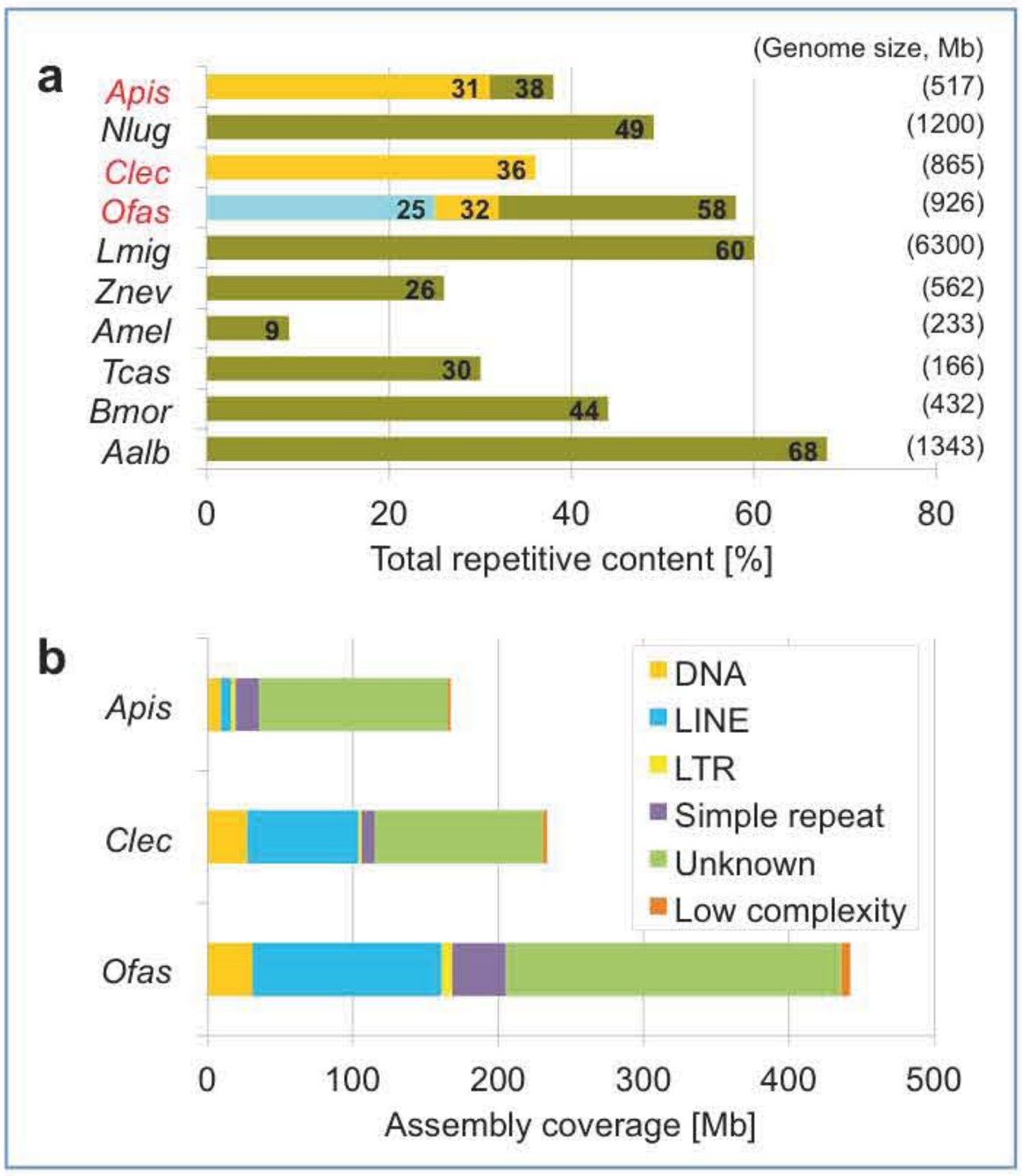


Fig 5. Comparison of repeat content estimations.

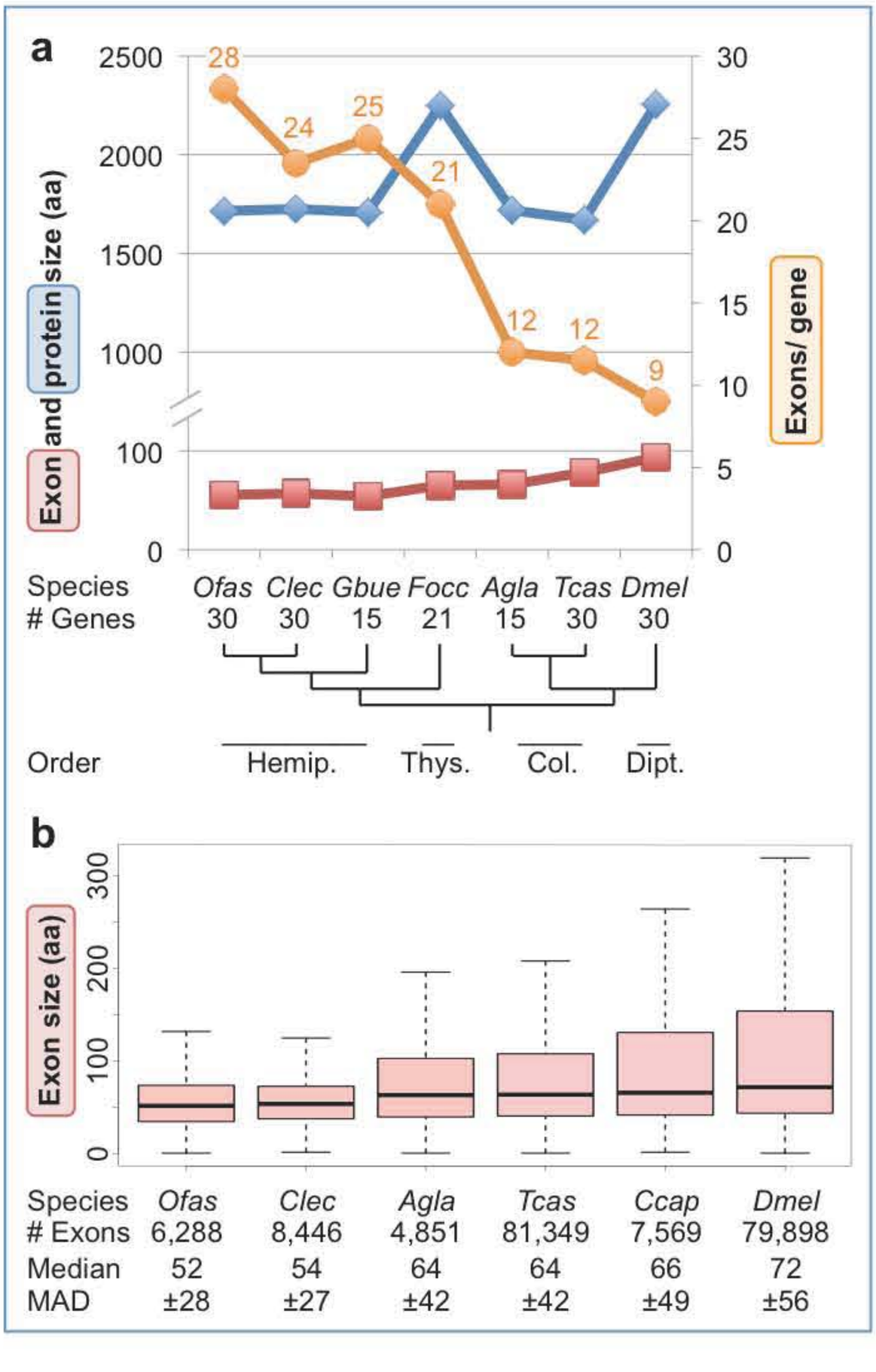


Fig 6. Trends in gene structure show hemipteroid-specific tendencies.

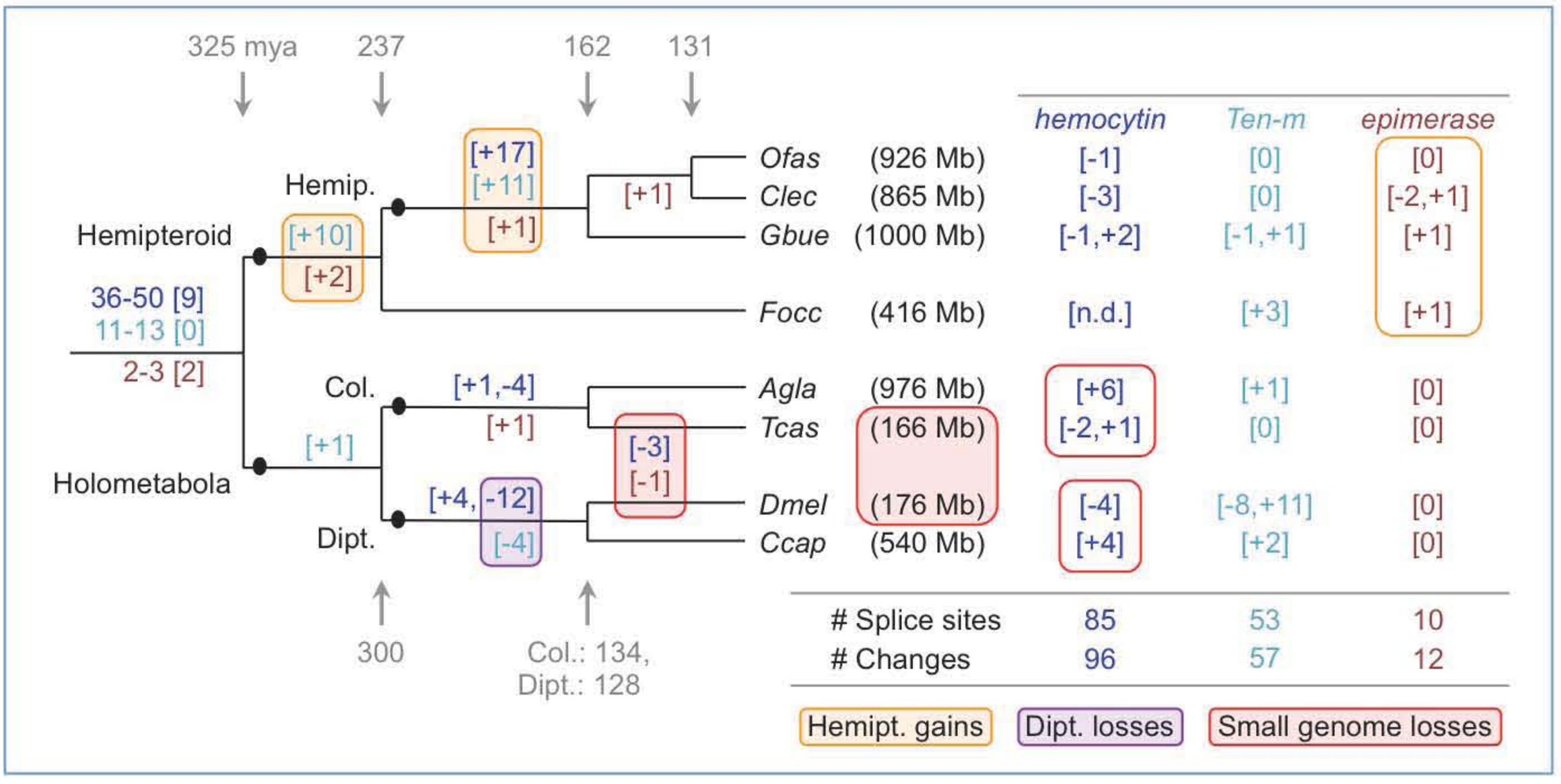


Fig 7. Splice site evolution correlates with both lineage and, independently, genome size.

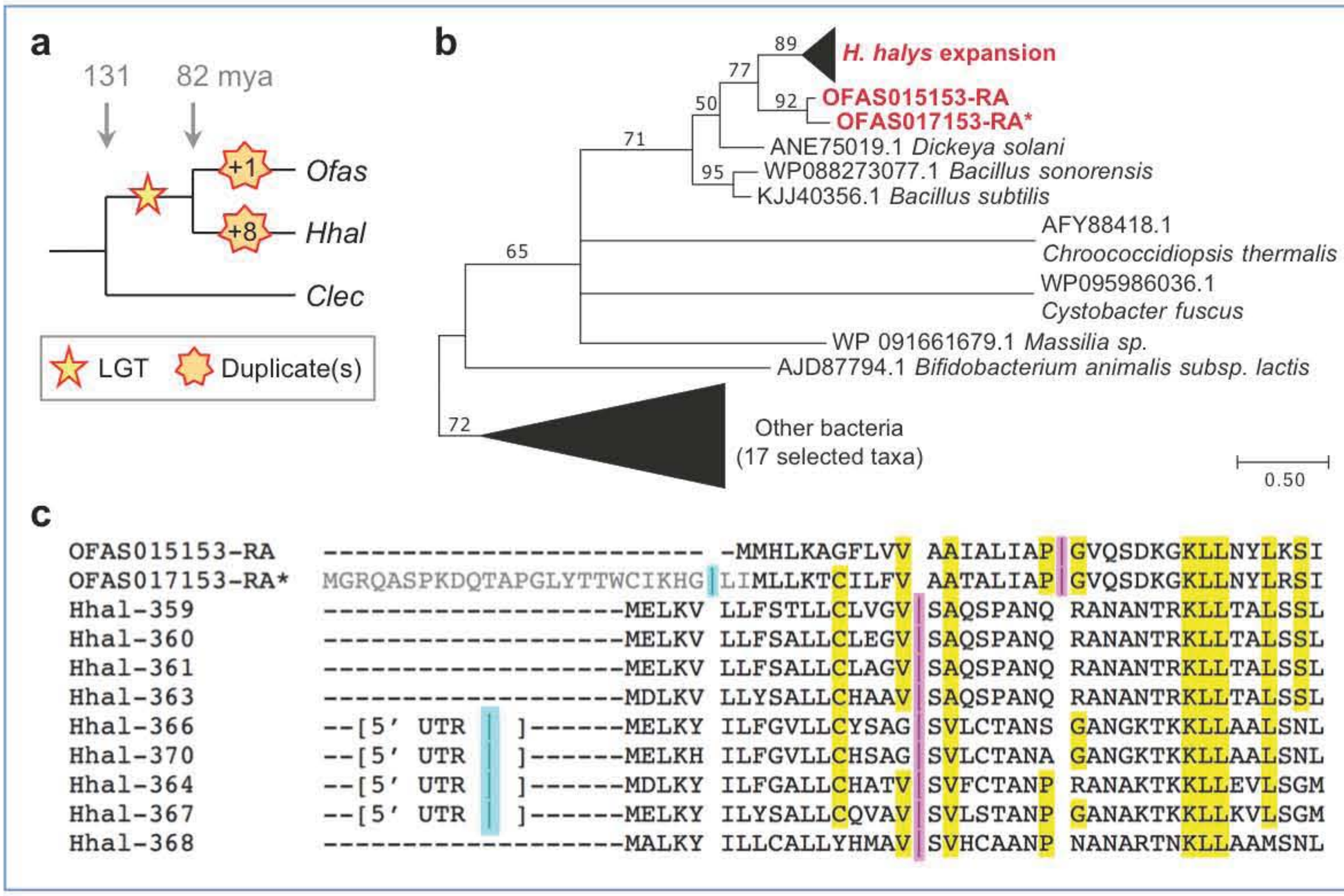
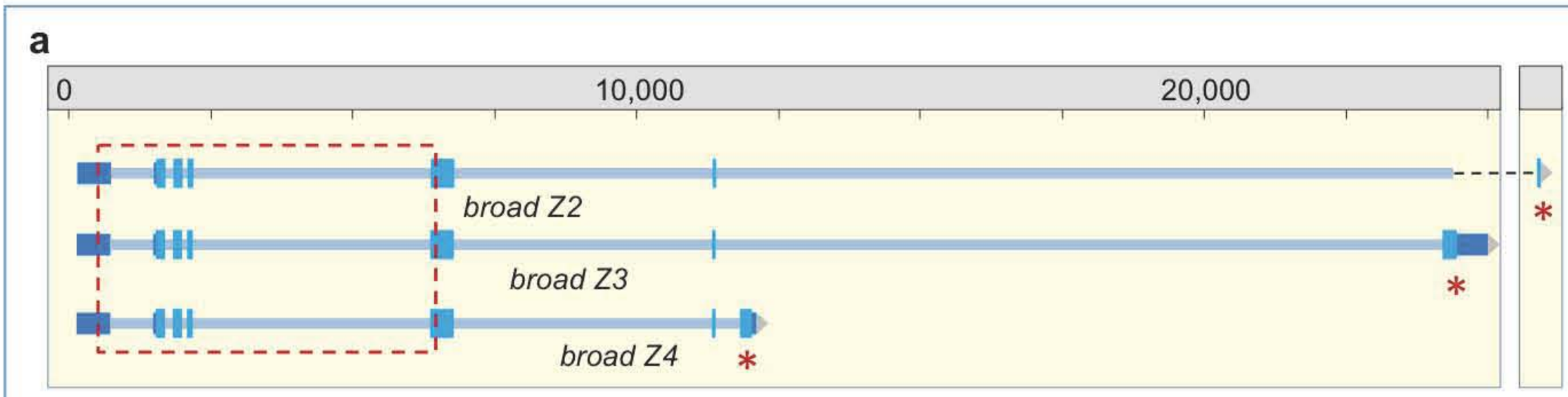


Fig 8. Lateral gene transfer introduction and subsequent evolution within the Hemiptera for mannosidase-encoding genes.



Treatment	Injected Nymphs	Surviving Adults	RNAi phenotype
Z2	33	12 (36%)	8 (67%)
Z3	29	16 (55%)	9 (56%)
Z4	28	22 (79%)	0 (0%)
Control	12	11 (92%)	0 (0%)

Fig 9. Isoform-specific RNAi based on new genome annotations affects the molting and cuticle identity gene *broad*.

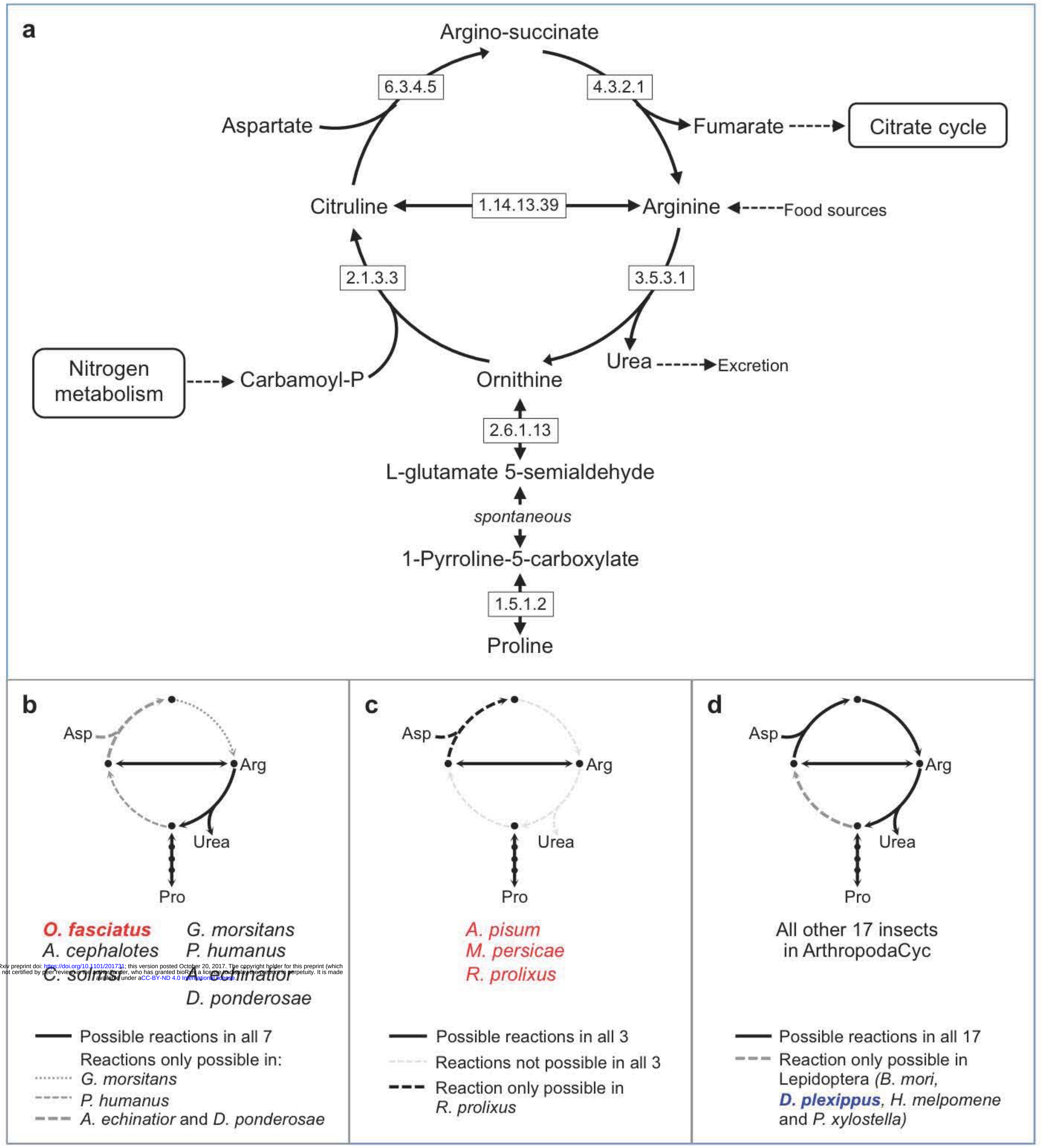


Fig 10. Comparison of the urea cycle of *Oncopeltus* with 26 other insect species.