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1 # Predictability in the evolution of Orthopteran cardenolide
2 # insensitivity

3

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1 **Abstract**

2 The repeated evolutionary specialisation of distantly related insects to cardenolide-containing
3 host plants provides a stunning example of parallel adaptation. Hundreds of herbivorous insect
4 species have independently evolved insensitivity to cardenolides, which are potent inhibitors of
5 the alpha-subunit of Na^+, K^+-ATPase (ATPα). Previous studies investigating ATPα-mediated
6 cardenolide insensitivity in five insect orders have revealed remarkably high levels of parallelism
7 in the evolution of this trait, including the frequent occurrence of parallel amino acid
8 substitutions at two sites and recurrent episodes of duplication followed by neo-functionalisation.
9 Here we add data for a sixth insect order, Orthoptera, which includes an ancient group of highly
10 aposematic cardenolide-sequestering grasshoppers in the family Pyrgomorphidae. We find that
11 Orthopterans exhibit largely predictable patterns of evolution of insensitivity established by
12 sampling other insect orders. Taken together the data lend further support to the proposal that
13 negative pleiotropic constraints are a key determinant in the evolution of cardenolide
14 insensitivity in insects. Furthermore, analysis of our expanded taxonomic survey implicates
15 positive selection acting on site 111 of cardenolide-sequestering species with a single-copy of
16 ATPα, and sites 115, 118 and 122 in lineages with neo-functionalised duplicate copies, all of
17 which are sites of frequent parallel amino acid substitution.
18

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1 **Introduction**

2 Two enduring fundamental questions in modern evolutionary biology are *what factors*
3 *limit the rate of adaptive evolution?* and *to what extent are adaptive evolutionary paths*
4 *predictable?* [1] Theoretically, the predictability of adaptation depends on a number of factors
5 that constrain the number of possible evolutionary paths. Among these is the number of potential
6 targets for beneficial mutations [2–4]. However, the extent to which adaptation is constrained by
7 mutation rate is unclear for most traits and some investigators have emphasised important roles
8 for pleiotropy, the phenomenon by which one mutation affects multiple phenotypes, and
9 epistasis, the effect of genetic background on the contribution of a mutation to a given
10 phenotype, among other factors [1,5–7].

11 Evaluating the relative importance of these factors has been challenging. One approach
12 has been to cobble together examples of adaptations from different traits in different species and
13 contexts in an attempt to come to general conclusions (e.g. [8,9]). However, the heterogeneity
14 inherent in such broad comparisons of different traits in different biological contexts may
15 substantially limit the power to make inferences from such data [10]. An alternative is to
16 examine cases on a trait-by-trait basis in the context of adaptation to common selective pressure
17 [10]. Instances of parallel evolution, the independent evolution of similar features in different
18 lineages, can provide multiple portraits of the evolutionary process and offer insight into the
19 factors that constrain adaptation and the extent to which adaptive evolutionary paths are
20 predictable.

21 Examples of parallelisms from nature are abundant and occur at different scales from the
22 resemblance of morphological traits to individual nucleotide substitutions that encode regulatory
23 or protein changes [4,11,12]. Such examples will have greater power to make inferences about
24 the factors determining the dynamics of adaptation for a given trait as the number of independent

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1 outcomes becomes larger, the more similar the selective pressure and the more is known about
2 the genetic basis of the underlying trait. With these factors in mind, one fertile area for
3 exploration is the repeated evolution of insensitivity of herbivorous insects to toxic secondary
4 plant compounds. Plants are ubiquitously equipped with secondary chemical defences such as
5 alkaloids, cyanogenic glucosides, and terpenoids that contribute to defence against herbivory
6 [13,14]. Despite these defences, herbivorous insects have in many cases repeatedly evolved
7 mechanisms to render them insensitive to toxic compounds [14,15], and even sequester toxins
8 for their own use [16].

9 A striking example is presented by herbivorous insects that have repeatedly evolved the
10 ability to feed on and, in many cases, sequester cardenolides from Apocynaceae plants, which
11 include milkweed [15]. Cardenolides represent a class of steroidal glycosides ("cardiac
12 glycosides") that bind to and inhibit the alpha-subunit of Na^+, K^+-ATPase (ATPα). This protein
13 is a ubiquitously distributed membrane-bound ion active-transporter present in animals with
14 well-known roles in a variety of physiological processes including neural signal transduction,
15 muscle contraction and osmoregulation [17]. Conservation of the cardenolide-binding domain of
16 ATPα among distantly-related animals, including vertebrates and invertebrates, suggests
17 important physiological roles for the regulation of Na^+, K^+-ATPase by endogenously produced
18 cardenolides [17,18]. In fact, cardenolides have been used medicinally for hundreds of years as
19 common treatments for conditions such as congestive heart failure and cardiac arrhythmias [18].
20 A growing number of studies have implicated the regulation of Na^+, K^+-ATPase by putatively
21 endogenous cardenolides in signalling pathways linked to a variety of pathologies including
22 hypertension and cancer [19,20].

23 Due to its medical importance, the interaction between Na^+, K^+-ATPase and cardenolides
24 has been well-studied. The binding of cardenolides arrests Na^+, K^+-ATPase in the

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1 phosphorylated state, where K^+ cannot be bound, Na^+ cannot be released to the extracellular side
2 and ATP is not hydrolysed [21]. Mutagenesis experiments, enzyme-ligand co-crystal structures,
3 and evolutionary analyses have implicated 41 amino acid residues of ATPα, scattered throughout
4 the protein, that either directly interact with cardenolides or affect their binding-affinity
5 indirectly (references listed in **Table S1**). These sites are largely concentrated near the site of
6 cardenolide binding in ATPα, with some exceptions (**Figure S1**). As such, the evolution of
7 cardenolide-insensitivity via the modification of ATPα (i.e. target-site insensitivity) is one of the
8 rare traits for which we have a good *a priori* idea of the beneficial mutation target size for
9 adaptation.
10 Broadly speaking, strategies employed by specialist herbivores to deal with toxin
11 compounds include destroying and/or excreting the toxins [22,23], inactivating the toxins by
12 chemical modifications [24], restricting the expression of the target protein to specific tissues
13 [25,26], and/or the evolution of target-site insensitivity [24]. The evolution of ATPα insensitivity
14 has so far been inferred in almost all Apocynaceae-specialist species surveyed from five insect
15 orders, including Lepidoptera, Diptera, Coleoptera, Hymenoptera, and Hemiptera [27–33].
16 Studies of the evolution of ATPα insensitivity in these five insect orders have revealed a
17 remarkable degree of convergence of molecular mechanism at multiple levels. First, despite the
18 identification of 41 residues in the protein that could potentially modulate sensitivity of ATPα to
19 cardenolides (**Table S1**), there is a marked enrichment of substitutions in Apocynaceae-
20 specialists observed at two sites in the protein, Q111 and N122, that flank the H1-H2
21 extracellular loop [30,33]; the substitutions Q111L, Q111T, Q111V and N122H occur in parallel
22 in multiple lineages. Second, several rounds of duplication of ATPα have occurred in parallel in
23 multiple species from three of the five surveyed insect orders, including Coleoptera, Hemiptera,
24 and Diptera [30,33]. In each case, at least one of the divergent copies harbours a number of

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1 cardenolide insensitivity-conferring substitutions [30,33,34]. Third, in most cases of ATPα
2 duplication, the copies have been shown to exhibit parallel evolution of tissue-specific
3 expression patterns, with putatively less cardenolide-sensitive copies predominating in the gut,
4 and putatively more sensitive copies predominating in nervous tissue [30].
5 Taken together, these data suggest a key role for negative pleiotropy in the evolution of
6 cardenolide-insensitivity in Apocynaceae-specialists: those species with a single copy are largely
7 limited to evolution at just a few of the 41 possible sites, but those with duplicates can explore
8 many other evolutionary paths using one of the differentially expressed duplicate copies [30].
9 Further support for the idea that negative pleiotropy plays a key role comes from site-directed
10 mutagenesis studies demonstrating trade-offs in Na^+, K^+-ATPase function (e.g. efficiency of
11 ATP hydrolysis) associated with some duplicate-specific substitutions observed in milkweed
12 bugs (Hemiptera) [35].
13 The notion of predictability in evolution is often framed in terms of forecasting future
14 events, for example, predicting the next steps in virus evolution [36]. However, it can also be
15 used in the sense of looking at evolutionary patterns retrospectively and asking if a set of rules
16 deduced from patterns of evolution in one group of organisms for a particular adaptation can
17 reliably predict the genetic architecture of the same trait in another group. With this in mind, we
18 survey a sixth insect order, the Orthoptera, which is phylogenetically positioned outside of the
19 five insect orders that have previously been investigated (**Figure 1A**). Within Orthoptera, we
20 focus on the family Pyrgomorphidae, commonly known as gaudy grasshoppers. This group is a
21 relatively small group of approximately 500 species that include some of the most colourful and
22 showy grasshoppers in the world (**Figure 1B-D**) [37]. Some members of this family that are
23 known to feed on toxic plants, including Apocynaceae, possess aposematic colouration and are
24 able to sequester plant secondary metabolites such as cardenolides and pyrrolizidine alkaloids

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1 [38–41]. In addition to the aposematic colouration, some genera (such as *Phymateus,*
2 *Poekilocerus, Zonocerus*) possess a unique mid-dorsal abdominal gland capable of squirting
3 toxic chemical when disturbed, while others (*Aularches, Dictyophorus, Taphronota*) can produce
4 foam as a result of haemolymph released through pores combined with air [41–44].
5 Studies of the common milkweed grasshopper *Poekilocerus bufonius* demonstrated that
6 they were substantially less sensitive to the cardenolide ouabain injections (as measured by
7 LD50) than species that do not feed on milkweed plants [45]. In addition, enzyme inhibition
8 assays performed on extracts from *P. bufonius* suggest cardenolide insensitivity of Na^+, K^+-
9 ATPase. In addition, observed heterogeneity among tissues in the degree of cardenolide-
10 insensitivity was interpreted as possible evidence for distinct isoforms of the enzyme in this
11 species [45]. While several species within Pyrgomorphidae show plant-mediated chemical
12 defence, most of the species in the family do not possess aposematic colouration or feed on toxic
13 plants [37], which presents an interesting opportunity to investigate the variation in cardenolide-
14 insensitivity of ATPα.
15 To shed light on the evolution of cardenolide-insensitivity in Pyrgomorphidae, we
16 generated RNA-Seq data for one representative grasshopper species from each of ten genera in
17 the family and reconstructed the ATPα by *de novo* transcriptomic assembly [30]. We find
18 remarkably similar patterns of amino acid substitution to those observed in previous surveys of
19 Apocynaceae-specialist herbivores, including a duplication event followed by neo-
20 functionalisation and tissue-specific differential expression in the genera *Phymateus* and
21 *Poekilocerus*. This expanded dataset, now including data for 52 Apocynaceae-feeding species
22 from six insect orders, further supports the view that adaptation in this system appears to be
23 largely constrained by negative pleiotropic effects associated with otherwise adaptive

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1 substitutions. The dataset also affords increased power to detect positive selection acting on
2 specific sites in the protein that are also sites of recurrent parallel amino acid substitution.
3
4 **Materials and Methods**
5 **Sequencing and *de novo* transcriptome assembly**
6 For details on sample collection and preparation see **Table S2**. Dissections were carried
7 out in Phosphate-buffered saline solution and stored either in TRIzol (Ambion, Life
8 Technologies) or RNAlater (Ambion Inc.) at -80ºC. For all insects, total RNA was extracted
9 using TRIzol (Ambion, Life Technologies) following the manufacturer's protocol. RNA-seq
10 libraries of *Aularches miliaris* and *Poekilocerus pictus* were prepared with TruSeq Stranded
11 total RNA Library Prep Kit with Ribo-Zero Gold (Illumina) and sequenced on Illumina
12 HiSeq2500 at AgriGenome (Cochin, Kerala, India). The libraries of *Taphronota calliparea* and
13 *Dictyophorus griseus* were prepared with NEBNext Ultra RNA library Preparation Kit (NEB)
14 and sequenced on Illumina HiSeq4000 (Genewiz, South Plainfield, NJ, USA). The libraries of
15 *Chrotogonus hemipterus*, *Atractomorpha acutipennis*, *Zonocerus elegans*, *Phymateus leprosus*,
16 *Ochrophlebia cafra*, and *Sphenarium purpurascens* were prepared with TruSeq RNA Library
17 Prep Kit v2 (Illumina) and sequenced either on Illumina HiSeq4000 (Genewiz, South Plainfield,
18 NJ, USA) or HiSeq2500 (Genomics Core Facility, Princeton, NJ, USA). Reads were trimmed
19 for adapters, quality and length (Phred quality ≥20 and ≥30 contiguous bases) using
20 TQSfastq.py
21 (http://code.google.com/p/ngopt/source/browse/trunk/SSPACE/tools/TQSfastq.py). All
22 transcriptomes were *de novo* assembled with Trinity 2.2.0 [46]. ATPα of *Locusta migratoria*
23 (GenBank: KF813097.1) was used to query the assembled transcripts using BLAST (blast-

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1 2.26). Reconstructions of ATPα for each species were used iteratively as query sequences to
2 BLAST against each other using either tblastx or blastn to recover all ATPα copies.
3 We have also included previously unpublished full-length ATPα sequences for a number
4 of other Apocynaceae-specialists including *Daphnis nerii* (Oleander Hawk-moth, Lepidoptera),
5 *Empyreuma pugione* (Spotted Oleander Caterpillar Moth, Lepidoptera), *Euploea core* (the
6 Common Crow, Lepidoptera), *Danaus chrysippus* (Plain Tiger, Lepidoptera), *Liriomyza*
7 *asclepiadis* (Milkweed Leaf-Miner Fly, Diptera). The methods to reconstruct these sequences is
8 identical to those used above following Zhen *et al*. [30]. Particular attention is paid to 41 sites
9 implicated in cardenolide-insensitivity (**Table S1**) established based on site-directed mutagenesis
10 and protein-ligand co-crystal structure studies.
11
12 **Discovery and confirmation of duplicates**
13 Given previous studies revealing duplications of ATPα associated with Apocynaceae-
14 specialisation [30], we evaluated evidence for duplication in our Orthopteran species data. Two
15 ancient lineages of ATPα (ATPα1 and ATPα2) precede the diversification of multiple insect
16 orders and form a distinct clade from the duplications of ATP1A found in vertebrates [30].
17 However, the expression level of ATPα2 is low in insects surveyed to date and its function
18 remains largely unknown. In *Drosophila melanogaster*, expression of ATPα2 is limited to larval
19 imaginal discs and adult male testes and accessory glands; RNAi and P-element knock-outs of
20 the gene are homozygous viable but male-sterile (http://www.flybase.org/reports/FBgn0267363).
21 Furthermore, we failed to recover an ortholog of ATPα2 from the *Locusta migratoria* genome
22 assembly [47], and several other Orthopteran assemblies [48,49], suggesting that the ATPα1/α2
23 duplication may have arisen after the split of Orthopterans from the other insects we have
24 surveyed (**Figure 1A**). We thus decided to focus on ATPα1, which shows clear orthology across

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1 taxa and a strong correlation between evolution at known cardenolide-sensitivity sites in the
2 protein and specialisation on cardenolide-containing Apocynaceae plants [30].
3 The discovery of duplicated copies of ATPα1 in *Poekilocerus pictus* and *Phymateus*
4 *leprosus* was verified by cloning and sequencing. Total RNA was extracted as described above,
5 and reverse-transcribed to single-strand cDNA using SuperScript® III Reverse Transcriptase
6 (Thermo Fisher Scientific). ATPα1 was PCR-amplified using Phusion High-Fidelity DNA
7 Polymerase (Thermo Fisher Scientific) using forward primer: 5'-
8 ACATGGCGGCAAGAAGAAAG-3' and reverse primer: 5'-
9 AGTAGGGGAAGGCACAGAAC-3'. The PCR product was cleaned using QIAquick PCR
10 Purification Kit (Qiagen), 3'A-tailed using Taq polymerase (NEB) and cloned into TOPO TA
11 vector (Invitrogen) following the manufacturer's instructions. Ampicillin-resistant colonies were
12 picked and screened by colony-PCR for the presence of inserts on a 1% agarose gel. Libraries of
13 plasmids were constructed using Tn5 transposase [50] annealed with Tn5ME-A, 5'-
14 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3' and Tn5ME-B, 5'-
15 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3' and indexed with customised
16 Illumina i7, i5. Paired-end 150 nt reads were collected for the pooled library on an Illumina
17 MiSeq Nano (Genomics Core Facility, Princeton University). *De novo* transcriptome assembly
18 was performed with Velvet/Oases [51,52] using a random sub-sample of 10,000 reads for each
19 indexed plasmid.
20
21 **Differential expression analysis**
22 Head, muscle, and foregut tissues of three male and three female *Poekilocerus pictus*
23 were dissected and sequenced in two batches (see sample collection and sequencing). Adapter
24 and quality trimmed reads were mapped back to our *de novo* assembly of the transcriptome as a

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1 reference (including ATPα1A and ATPα1B) using bwa mem [53] with default criteria, processed
2 with SAMtools 0.1.18 [54] and mapped reads were counted with HTSeq 0.6.1 [55]. We used the
3 inverted beta-binomial (ibb) test [56] to determine the significance of difference of expression
4 level between tissues. The method uses a negative binomial distribution in a generalised linear
5 model framework for paired-sample testing. We applied a standard Bonferroni correction to
6 account for multiple tests. Paired-sample count data were normalised by either total number of
7 mapped reads or the sum of reads mapping to ATPα1A and ATPα1B (**Table S3**).
8
9 **Re-analysis of Lygaeid ATPα1 evolution and expression.**
10 Using the recently completed *Oncopeltus fasciatus* genome [57], we detected a fourth
11 copy of ATPα1 (ATPα1D) in these Lygaeid bugs that was missed by previous studies. Lygaeid
12 ATPα1D is the least-derived copy at sites implicated in cardenolide-sensitivity and is expressed
13 in *O. fasciatus* heads, yet has the lowest expression of the four copies (**Figure 3A**). We also
14 confirmed that, like copies A-C, ATPα1D is also shared with *Lygaeus kalmii* (a sister-genus
15 species) but we could only partially reconstruct it from our *de novo* transcriptome assembly due
16 to its low expression. Using RNA-seq data for *O. fasciatus*, we confirmed the finding of
17 differential expression of duplicates documented by Zhen *et al.* [30] **(Figure S3A)**. All four
18 copies of ATPα1 in the Lygaeid *O. fasciatus* are more highly expressed in the head than the gut.
19 The putatively most sensitive copy (ATPα1D), despite having the lowest expression level of the
20 four copies, exhibits the greatest degree of up-regulation in the head relative to other copies
21 (**Figure S3B**).
22
23 **Evolutionary analyses**
24 The ages of the duplicates were calculated from dS (the per site rates of substitution at

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1 synonymous sites) estimated using PAML4.8 codeml [58], with prior trees based on established
2 cladistic relationships (Mariño-Pérez *et al*, unpublished, [59])and calibrated with divergence
3 times obtained from www.timetree.org (*Locusta* and Pyrgomorphidae: 117.4 Mya; *Napomyza*
4 and *Phytomyza*: 39 Mya). The divergence times of the Large Milkweed Bug (*Oncopeltus*
5 *fasciatus*), Milkweed Stem Weevil (*Rhyssomatus lineaticollis*), and Dogbane Beetle (*Chrysochus*
6 *auratus*) are taken from [30] where similar methods were used to date duplicates. In the Lygaeid
7 bugs (*O. fasciatus* and *L. kalmii)*, a phylogenetic analysis strongly suggests a duplication order
8 ((ABC),D), ((AB),C) and most recently (A,B).

9 To obtain distributions of lineage-specific evolutionary rates, ATPα1 lineages were
10 grouped into four categories. ATPα1 of all non-specialist species were denoted as "Outgroup".
11 ATPα1 of Apocynaceae-specialists with a single copy of ATPα1 are denoted "Single". For
12 specialists with multiple copies of ATPα1, copies that are up-regulated in the gut relative to the
13 head are assumed to be relatively cardenolide-insensitive copies and marked as Dup^{I} [30].
14 Likewise, those up-regulated in the head relative to the gut are assumed to be relatively sensitive
15 copies and were grouped as Dup^{S}. We chose this criterion rather than the number of
16 insensitivity-conferring substitutions because using the latter makes the designation of copies
17 with intermediate numbers of substitutions ambiguous. In Lygaeid bugs (*O. fasciatus* and *L.*
18 *kalmii)*, this implies that copies A and B are treated as relatively insensitive copies (Dup^{I}),
19 whereas C and D are treated as relatively sensitive (Dup^{S}) (**Figure S3**). The dN/dS ratios
20 (omega) for ATPα1 along a lineage were estimated within each insect order using PAML codeml
21 under free ratio model. Parameters were set as follows: seqtype = 1, model = 1, NSsites = 0,
22 clock = 0, CodonFreq = 2, fix_kappa = 0, kappa = 2.0, fix_omega = 0, omega = 0.02.
23 Differences in the distribution of branch-specific estimates of omega between each group were
24 tested with Dunn's test of multiple comparisons using rank sums as implement in R (dunn.test).

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1 We also evaluated evidence for positive selection acting on individual sites of ATPα1
2 using PAML codeml. To do this, we defined and investigated several models (**Figure 7, Table**
3 **S4**). Model 1: positive selection on all Apocynaceae-specialist lineages; Model 2: positive
4 selection on all Apocynaceae-specialist lineages with single copies of ATPα1 (including
5 ancestral lineages prior to duplication); Model 3: positive selection on Dup^S and Dup^I lineages of
6 Apocynaceae-specialists; Model 4: positive selection on Dup^I lineages of Apocynaceae-
7 specialists; Model 5: positive selection on all outgroup lineages. Tests were carried out using the
8 modified branch-site model A implemented in codeml [60–62] with parameters set as follows:
9 seqtype = 1, model = 2, NSsites = 0, clock = 0, CodonFreq = 2, fix_kappa = 0, kappa = 2.0,
10 fix_omega = 0, omega = 0.02. The ancestral sequences of duplicated ATPα1 were reconstructed
11 with the function RateAncestor. Unrooted trees were used, and branch labels were added
12 manually for each model. The method assigns each codon a Bayes Empirical Bayes (BEB)
13 posterior probability that the codon belongs to a site class with omega > 1 (i.e. indicating
14 positive selection). A BEB posterior probability of >0.95 was considered evidence for positive
15 selection.
16
17 **Results**
18 **Survey of ATPα1 of 15 Orthopteran genera**
19 Using an RNA-seq-based gene discovery method, we reconstructed the complete coding
20 sequences of the alpha subunit of Na^+, K^+-ATPase (ATPα1) of grasshoppers from species
21 representing ten genera in the family Pyrgomorphidae, as well as five outgroup species within,
22 and one outside, the order Orthoptera (**Figure 2**). Our broad survey of Apocynaceae-feeding
23 Pyrgomorphidae revealed few amino acid substitutions among the 41 sites implicated in
24 cardenolide-sensitivity (**Table S1**). The two most broadly distributed substitutions, Q111L and

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1 A119S, correlate only weakly with Apocynaceae-feeding, aposematism, and the presence of
2 abdominal defensive glands in the group. The glaring exception is the lineage leading to the
3 genera *Poekilocerus* and *Phymateus*, both containing multiple species, which appear to share a
4 duplication of ATPα1. Both species surveyed retain an ancestral version of the protein
5 (ATPα1B) while having a highly-derived copy (ATPα1A). The diverged ATPα1A copies of
6 *Poekilocerus* and *Phymateus* share many amino acid substitutions relative to the ancestral copy,
7 several at sites implicated in cardenolide-sensitivity. Phylogenetic analysis clearly indicates that
8 the duplication of ATPα1 and functional divergence of ATPα1A predates the diversification of
9 these clades into separate genera and species and we estimate the age of the duplication to be ~36
10 million years old.
11

12 **Patterns of amino acid substitution in ATPα1 of Orthopterans**

13 Cross-referencing the pattern in Orthopterans with other Apocynaceae-specialists
14 surveyed in other insect orders reveals a high level of parallel amino acid substitution (**Figure 3,**
15 **Figure S2**). Conspicuous among these are two parallel substitutions Q111L and A119S, which
16 appear to pre-date the diversification of the Pyrgomorphidae. Cell transfection experiments have
17 shown when substitutions at position 111 are introduced to a sensitive background of the *D.*
18 *melanogaster* protein, the survival rate of HeLa cells increases 3 to 8-fold [31]. Interestingly,
19 A119S is observed in almost every Apocynaceae-specialist species that has been surveyed to
20 date (**Figure S2**), including the *Drosophila subobscura* subgroup where resistant forms of
21 ATPα1 have been documented segregating as polymorphisms within *D. subobscura* [63].
22 Exceptions include aphids (*Aphis nerii* and *Acrythosiphon pisum*), the milkweed leaf beetle
23 (*Labidomera clivicolis*) and several Hymenopteran species that harbour the similar substitution,
24 A119N. Q111L and A119S are not associated with cardenolide-feeding or Apocynaceae-

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1 specialisation in the Orthoptera and are possessed by a number of non-aposematic species not
2 known to feed on cardenolide-containing plants (e.g. *L. migratoria*, *C. hemipterus*, *A.*
3 *acutipennis*). The substitutions Q111L, A119S, and A119N also occur sporadically among a
4 number of other insects not known to feed on Apocynaceae (**Figure S2**).
5 Considering the cardenolide-insensitive ATPα1A copy-specific substitutions of
6 *Poekilocerus* and *Phymateus*, N122H stands out as a substitution observed in parallel in many
7 other Apocynaceae-specialists including at least one member of each of the six insect orders
8 surveyed. The N122H substitution in isolation has been shown to increase *Drosophila* ATPα1
9 insensitivity to the cardenolide ouabain by 250-fold [64] and increase survival of HeLa cells
10 challenged with ouabain [31]. N122H has also been reported to interact synergistically with
11 substitutions at Q111, though Q111L was not tested [31]. The Orthopteran ATPα1A copy-
12 specific substitutions V115T, P118S, D121N, I315V and L874M also occur in at least one other
13 insect order. Of these, D121N is known to decrease cardenolide-sensitivity by ~100-fold [65].
14
15 **Unique substitutions associated with duplication of ATPα1 and differential expression of**
16 **neo-functionalised copies.**
17 Using data from three insect orders, Zhen *et al.* [30] found a significant enrichment of
18 unique substitutions at sites implicated in cardenolide-sensitivity in Apocynaceae-specialist
19 lineages with duplicated copies of ATPα1 compared to those that retain a single copy. They also
20 documented convergent patterns of differential gene expression of independently derived
21 duplicates found in specialists. Specifically, they noted that duplicates inferred to be the most
22 sensitive to cardenolides, based on the number of substitutions at sites implicated in cardenolide-
23 insensitivity, consistently exhibited up-regulation in the head relative to the gut. Zhen *et al*. [30]
24 argued that this might be expected since the gut is the site of first-processing of cardenolides and

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1 sensitive forms of ATPα1 in nervous tissue are likely protected to some extent by the blood-brain
2 barrier provided by the glial sheath surrounding neurons [30,66]. Zhen *et al.* [30] interpreted this
3 pattern as being consistent with a key role for pleiotropy in the evolution of cardenolide-
4 insensitivity. Specifically, they proposed that there might be trade-offs in enzyme performance
5 associated with unique substitutions that are ameliorated by differential expression of neo-
6 functionalised duplicate copies. Here, we re-evaluate this claim in the context of our expanded
7 dataset, which now includes Orthoptera and previously published data for five other insect
8 orders.

9 Despite the remarkable parallelism at the levels of gene duplication and amino acid
10 substitutions, it is notable that “unique” substitutions (i.e. those unique to one lineage) at sites
11 implicated in cardenolide-sensitivity in Orthoptera are restricted to duplicated copies, as
12 observed in other Apocynaceae-specialists with duplications. Notably, ATPα1A of *Poekilocerus*
13 and *Phymateus* share a unique two amino acid insertion between residues 119 and 120 of the
14 ancestral protein (**Figure 2**). Insertions or deletions have not been observed so far among the
15 other five insect orders surveyed to date (**Figure 3, Figure S2**). Furthermore, *Phymateus*
16 *leprosus* harbours an additional amino acid substitution (D120E) that also appears to be unique
17 among Apocynaceae-specialists. Notably, no unique substitutions were observed among the
18 other 12 Orthopteran species surveyed that appear to retain a single copy of ATPα1.

19 We also find that the functionally diverged duplicates of ATPα1 in the Orthopteran
20 *Poekilocerus pictus* are differentially expressed in a similar manner to those found in Lygaeid
21 bugs (see Methods) and other Apocynaceae-specialists [30]. Specifically, the putatively less
22 cardenolide-sensitive copy (ATPα1A) is up-regulated relative to the more sensitive copy
23 (ATPα1B) in the gut compared to muscle and head (**Figure 4**). The cardenolide-sensitive copy
24 ATPα1B accounts for 18.7% of total ATPα1 expression in the brain but only 2.2% in the foregut,

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1 which is the primary location of cardenolide processing. This finding is consistent with previous
2 studies of the related *Poekilocerus* species, *P. bufonius*, that noted Na^+, K^+-ATPase activity of
3 the brain was more sensitive to cardenolide-inhibition than that of the gut [45]. Since the
4 duplication of ATPα1 appears to predate the diversification of *Poekilocerus*, it is highly likely
5 that the tissue-specific ouabain-sensitivity can be explained by the presence of the same
6 duplication we have documented here.

7 Having established tissue-specific expression of duplicate copies in Lygaeids (see
8 Methods) and Orthopterans, we then re-visited the pattern of unique versus parallel substitution
9 with respect to duplication status and differential expression of ATPα1 in the full dataset now
10 spanning six insect orders (**Figure 5**). Consistent with Zhen *et al*. [30], we find a marked
11 enrichment of unique substitutions at sites implicated in cardenolide-sensitivity in Apocynaceae-
12 specialists with duplicated copies of ATPα1 (Fisher's exact test P=0.0022). Examining the
13 pattern in more detail, it is apparent that unique substitutions appear to be unequally distributed
14 among copies. In each case of duplication, we can distinguish between less-sensitive and more-
15 sensitive copies based on the number of derived amino acid-substitutions that have been
16 implicated in cardenolide-sensitivity. We find a significant enrichment of unique substitutions in
17 the dataset (13/14) occur on putatively less-sensitive copies of ATPα1 that are substantially more
18 expressed in the gut than more-sensitive copies (in the 4/5 cases where expression patterns have
19 been investigated).

20

21 **Relaxed constraints on ATPα1 duplicates and positive selection for insensitivity.**

22 It is clear from the above analyses that the evolution of cardenolide-insensitivity in some
23 taxa is facilitated by duplication and differential expression of ATPα1, which is expected to relax
24 constraints at sites known to confer insensitivity but are associated with negative pleiotropic

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1 effects. We further carried out a phylogenetic analysis to ask 1) whether this relaxation in
2 constraint extends beyond sites directly implicated in cardenolide insensitivity, and 2) whether
3 there is evidence for positive selection associated with Apocynaceae-specialisation at these and
4 other sites in the protein. To examine patterns of constraint in more detail, we grouped both
5 external and internal branches of the ATPα1 phylogeny into four categories: Outgroup;
6 Apocynaceae-specialist lineages with a single ATPα1 copy (Single), and Apocynaceae-specialist
7 lineages harbouring duplications that are inferred to be either relatively sensitive (Dup^S), or
8 relatively insensitive (Dup^I) to cardenolides (see Methods). Examining the distributions of
9 omega (dN/dS) estimates among these classes reveals that putatively less sensitive copies of
10 ATPα1 (Dup^I) evolve ~5-fold faster than their more sensitive counterparts (**Figure 6**). We find
11 that this pattern persists if we exclude the sites directly implicated in cardenolide-sensitivity
12 (**Figure S4**), implying that relaxation of constraint on derived copies extends beyond this class of
13 sites in the protein.

14 We next asked whether relaxed constraint in Apocynaceae-feeding lineages, or on
15 insensitive duplicate ATPα1 lineages, is sufficient to account for the data or whether there is
16 evidence for positive selection associated with insensitivity-conferring amino acid substitutions.
17 We conducted a site-specific scan for positively selected substitutions using the improved branch
18 site model implemented in PAML (see Methods). Site 111, a site directly implicated in
19 cardenolide insensitivity and the target of frequent parallel amino acid substitution across insect
20 orders, is identified as positively selected in this analysis under models assuming either positive
21 selection in Apocynaceae-specialists, or only on Apocynaceae-specialist lineages bearing a
22 single copy of ATPα1 (BEB posterior probability ≈ 1.0 under both models, **Figure 7**, **Table S4**).
23 Sites 115, 118 and 122, also directly implicated in cardenolide-sensitivity, are identified as
24 positively selected under models of positive selection on the Dup^I lineages of ATPα1 (BEB

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1 posterior probabilities of 0.956, 0.998 and 0.993, respectively, **Figure 7**). Interestingly, evidence
2 for positive selection also emerges at several sites in the protein not previously implicated in
3 cardenolide-sensitivity. Seven sites show evidence for positive selection in lineages bearing
4 duplicate copies of ATPα1 including a cluster of three sites (560, 563, and 566) that are located
5 far from the cardenolide-binding domain (**Table S4, Figure S1**).
6
7 **Discussion**

8 Predictability in evolutionary biology not only refers to being able to forecast future
9 evolutionary events but is also a statement about the ability to predict the genetic basis of
10 adaptations outside a taxonomic group in which the rules governing the genetic basis of a
11 particular adaptation were deduced. Previous surveys of ATPα1 in the context of insect
12 adaptation to cardenolides established that, despite a reasonably large target size for evolving
13 target-site insensitivity (i.e. 41 sites implicated in cardenolide-sensitivity, **Table S1**), a small
14 proportion of these sites (and sites 111 and 122 in particular) are disproportionately used in the
15 evolution of insensitivity to cardenolides [30]. The exception to this pattern is found in insects
16 that have duplicated ATPα1 and differentially allocate a functionally-diverged copy to the gut,
17 while retaining an ancestral copy that is more highly expressed in the head.

18 To test the generality of these patterns as predictors, we surveyed aposematic
19 grasshoppers that sequester cardenolides belonging to Orthoptera, which is the first
20 Polyneopteran order to be examined for cardenolide insensitivity (**Figure 1**). Of the 10 species of
21 Pyrgomorphidae included in the analysis, six species have been reported to feed on Apocynaceae
22 and exhibit aposematic colouration and chemical defence (**Figure 2**). The remaining four species
23 feed on non-toxic plants and show cryptic colouration. While we initially expected all
24 Apocynaceae-feeding species to show substitutions at sites implicated in cardenolide-

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1 insensitivity, we found that most Orthopteran species harbour only two substitutions (Q111L and
2 A119S), that are not correlated with consumption and/or sequestration of cardenolide-containing
3 plants in this group. The specific substitution Q111L appears to only weakly confer insensitivity
4 to cardenolides based on previous functional studies. Furthermore, A119S does not interact with
5 cardenolides directly or have an effect on cardenolide-affinity, although it does contribute to
6 more rapid association/dissociation kinetics of the cardenolide ouabain [67]. Given this and their
7 broader phylogenetic distribution (**Figure 3**, **Figure S2**), it is possible that these ancient and
8 recurrent substitutions represent exaptations [68] that facilitate the evolution of more resistant
9 forms of ATPα1 in some insect lineages [29].

10 Nevertheless, two of the six species (*Poekilocerus* and *Phymateus*) do share a duplication
11 (ATPα1A) that exhibits multiple amino-acid substitutions known to confer insensitivity to
12 cardenolides. Why amino acid substitutions with large effects on cardenolide sensitivity or
13 insensitive duplicates of ATPα1 are not found in the other four Apocynaceae-feeding species
14 deserves an explanation. Of the six Apocynaceae-feeding species included in this study,
15 *Phymateus*, *Poekilocerus*, and *Zonocerus* have the mid-dorsal abdominal glands used for
16 chemical defence [41] and they form a monophyletic group within the phylogeny of
17 Pyrgomorphidae [37]. So far, cardenolides have been reported in the defence secretion from only
18 *Phymateus* and *Poekilocerus* [41], and their common names, milkweed grasshoppers, suggest a
19 strong association of these insects with the host plant. Although *Zonocerus* do feed on
20 cardenolide-containing plants, their main defence chemical is pyrrolizidine alkaloid [69–71],
21 rather than cardenolides. *Zonocerus variegatus* only excretes cardenolides when the toxins are
22 included in its diet [72]. The other three Apocynaceae-feeding species (*Aularches*, *Dictyophorus*,
23 and *Taphronota*) do not have specialised abdominal glands, but produce toxic foams through
24 various pores on the thorax and abdomen [41]. It is unknown whether they use cardenolides as

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1 their main chemical defence. The lack of cardenolide insensitivity of Na^+, K^+-ATPase in
2 *Zonocerus*, *Aularches*, *Dictyophorus*, and *Taphronota* seems to suggest that they rely on other
3 unknown mechanisms to cope with the toxicity of Apocynaceae. It is possible that while they are
4 capable of consuming Apocynaceae, they might prefer a mixture of other toxic plants containing
5 different kinds of secondary compounds to confer toxicity. Though the chemical ecology of these
6 insects is under-studied, we can postulate that, at least in Pyrgomorphidae, only those species
7 that are intimately associated with Apocynaceae have evolved the cardenolide insensitivity of
8 Na^+, K^+-ATPase.

9 Considering the full dataset now including data from six insect orders, our study confirms
10 previous findings [30] suggesting that, when ATPα1 has been duplicated, unique substitutions at
11 known functionally important sites are significantly enriched specifically among insensitive
12 copies (**Figure 5**). This pattern of relaxation of constraint associated with one copy is also
13 apparent at sites outside of those known to be functionally important sites (**Figure S4**). Both
14 patterns are a strong indication of neo-functionalisation rather than merely sub-functionalisation
15 of ATPα1. This may be expected given the age of the duplication event. Sub-functionalisation is
16 expected to occur rapidly after duplication while neo-functionalisation takes place over longer
17 timescales because it generally requires more mutations [73]. Most of the duplications of ATPα1
18 in Apocynaceae-specialist insects detected so far are indeed ancient and trans-specific (**Figure**
19 **3**).

20 Despite the expected signature of strong purifying selection on ATPα1 (**Figure 7**, **Table**
21 **S4**), we have also detected the signature of positive selection on sites implicated in cardenolide-
22 insensitivity and exhibiting frequent parallel substitution. Zou and Zhang [74] have pointed out
23 that the observation of parallel substitution *per se* is not sufficient to infer adaptive significance
24 since this pattern may be expected under a neutral model due to among-site differences in

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1 physicochemical constraints. Some evidence against this argument in the case of the evolution of
2 ATPα1 target-site insensitivity is provided by the fact that Apocynaceae-specialist species are
3 highly enriched for substitutions at sites implicated in cardenolide-sensitivity compared to non-
4 specialist outgroups [30; this study]. Our finding of positive selection at some of these sites
5 establishes a more direct link between adaptive protein evolution and recurrent parallel
6 substitutions at sites implicated in cardenolide-sensitivity.

7 Interestingly, we have also detected signatures of positive selection at sites not previously
8 implicated in cardenolide-sensitivity (**Table S4, Figure S1**). Some of these sites (e.g. 301, 560,
9 563, 566 and 667) are located far from the cardenolide-binding domain of the enzyme and sites
10 560, 563 and 566 are known to have roles in binding ATP. While this might seem to preclude
11 direct roles in cardenolide-insensitivity, the existence of sites exhibiting allosteric effects on
12 sensitivity is not unprecedented (e.g. 367 and 656, **Table S1**, [75]). This being said, the detection
13 of positive selection at these sites may reflect selection pressures that are either only indirectly
14 related or even unrelated to the evolution of cardenolide-insensitivity. Future functional
15 experiments could be aimed at understanding the effects of these substitutions on cardenolide-
16 insensitivity and overall enzyme performance.

17 Duplications of ATPα1 feature prominently in the evolution of cardenolide-insensitivity
18 in herbivorous insects. Given this feature, it will be interesting to compare patterns of recurrent
19 parallel amino acid substitution in insects with vertebrates. Bufonid toads are among the few
20 animals able to produce Na^+, K^+-ATPase-inhibiting compounds called "bufadienolides" that
21 closely resemble cardenolides and act in the same way [76]. As a result, predators of bufonid
22 toads represented by a wide variety of vertebrates are under pressure to evolve insensitivity to
23 these compounds [77,78]. In contrast to insects, vertebrates retain at least three copies of ATPα,
24 (ATP1A1, ATP1A2, and ATP1A3), that are differentially expressed among tissues [79].

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1 Previous studies have so far investigated the H1-H2 extracellular loop of ATP1A1 in bufonid
2 toads and predatory frogs [77] and ATP1A3 of a number of predatory squamates [78,80]. It will
3 be of considerable interest to further compare patterns of molecular evolution of cardenolide-
4 sequestering insects to their bufonid predator analogs in the context of complete reconstructions
5 of all three proteins.

6

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14

15 **Data accessibility**

16 All raw RNA-seq sequence data generated for this study have been deposited in the National
17 Center for Biotechnology Information Short Read Archive, www.ncbi.nlm.nih.gov/sra
18 (BioProject PRJNA509040). Sequences of ATPα1 used in our analysis have been submitted to
19 Genbank under Accession numbers MK294065-81.

20

21 **Author contributions**

22 L.Y. and P.A. designed the study; N.R., R.M-P, R.D., and K.K. provided the samples; L.Y.
23 performed the experimental work and analysed the data; M.W. contributed to Figure S3; A. R.

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1 contributed to Figure S2; L.Y. and P.A. wrote the manuscript; H.S., and R.M-P. reviewed and
2 edited.
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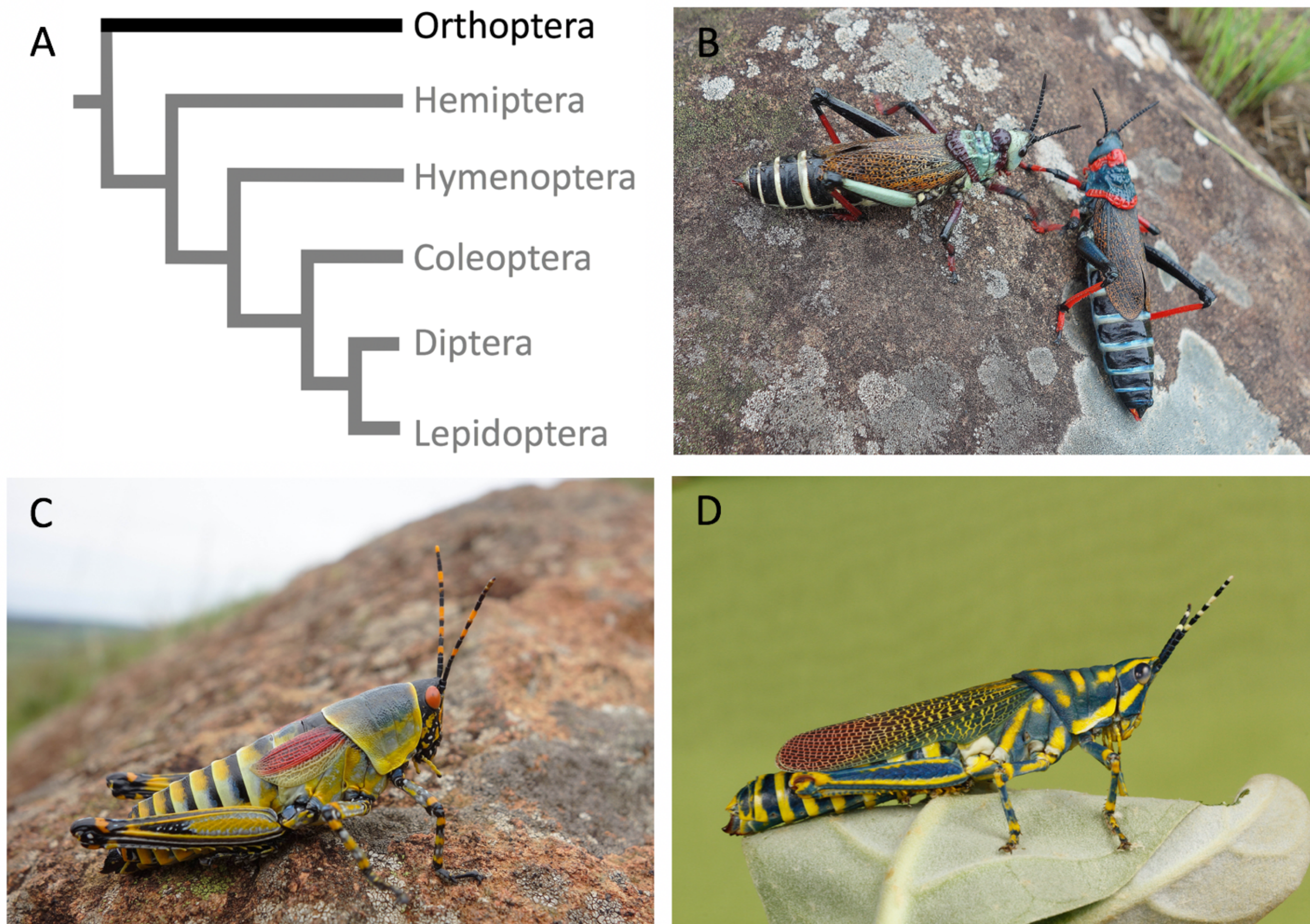

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3 **Figure 1**. Phylogeny (A) and representative members of the Orthoptera: Pyrgomorphidae (B-D).

4 Orthopteran species shown are B) *Dictyophorus spumans* (South Africa); C) *Zonocerus elegans*

5 (South Africa); D) *Poekilocerus pictus* (India).

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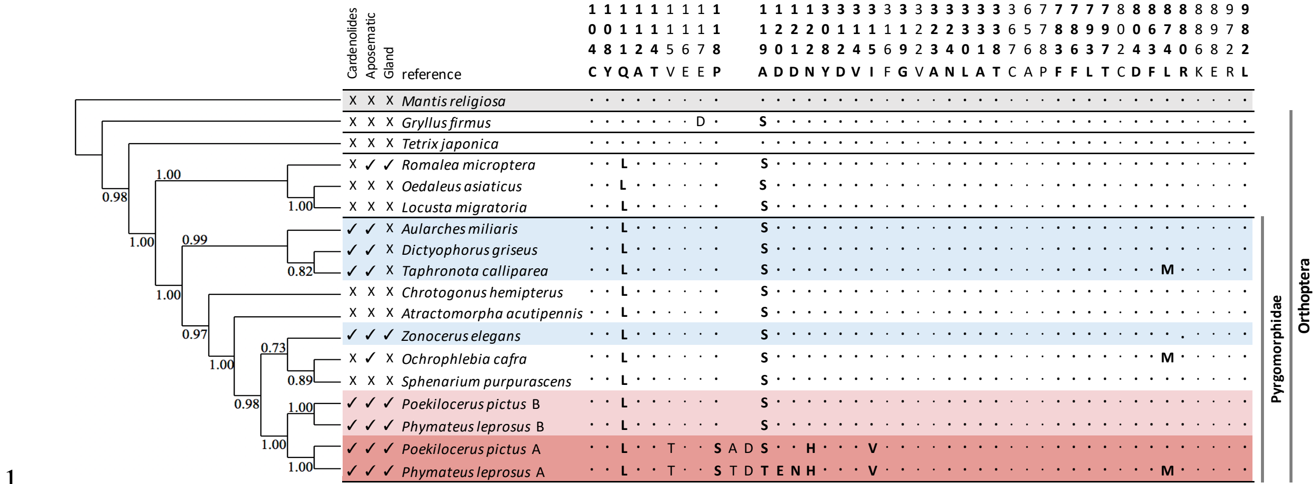

Figure 2. Amino acid substitutions at sites implicated in cardenolide-sensitivity for ATPα1 of Orthopterans in the family Pyrgomorphidae and outgroups. Each family is separated by black lines. The coloured rows correspond to putatively cardenolide-adapted species that either possess only one copy (light blue) or two copies (light/dark red) of ATPα1. The non-Orthopteran outgroup (*Mantis religiosa*, order Mantodea) is shaded in grey. The numbering of sites is based on sheep ATP1A1 (*Ovis aries*) (Genbank: NC019458.2). Bold columns correspond to the sites for which site-directed mutagenesis studies suggest a role in cardenolide-sensitivity, while the rest were identified by structural prediction. Dots indicate identity with the reference, which represents the consensus sequence among non-specialist Arthropods. Letters represent amino acid substitutions relative to the reference. The cladogram was constructed using a maximum likelihood method implemented in SeaView based on protein-coding nucleotide sequences of ATPα1 with bootstrap values shown. "Cardenolide" refers to species feeding on Apocynaceae. "Aposematic" refers to the presence of warning colouration patterns. "Gland" refers to the presence of a specialised abdominal defensive gland that secrete toxic chemicals.

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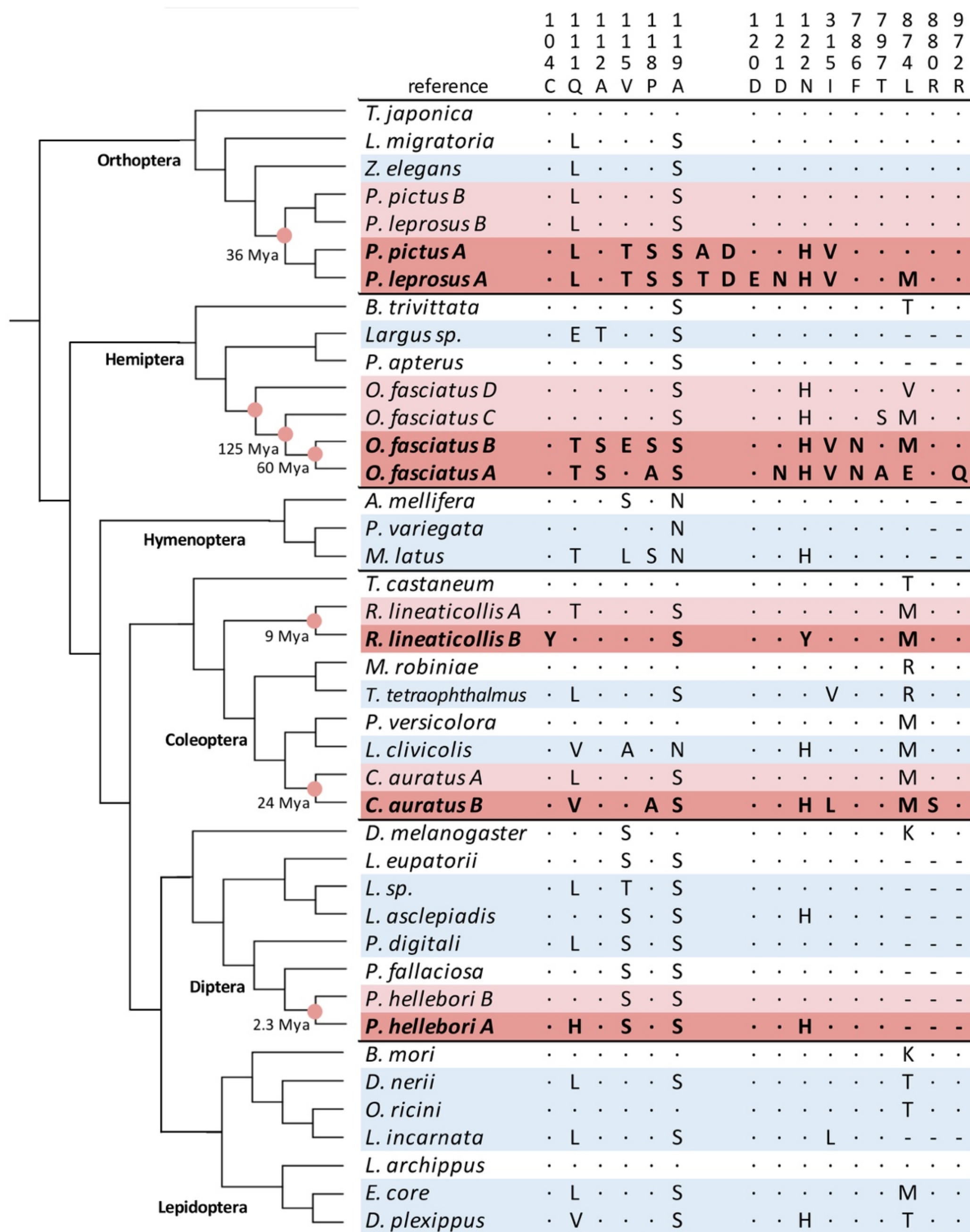

Figure 3. Patterns of amino acid substitution and duplication spanning six insect orders. Shown are sites implicated in cardenolide binding (**Table S1**) at which substitutions have occurred. The colour scheme is consistent with **Figure 2**. Red circles indicate duplication events. The cladogram represents the relationships of insect orders and species phylogenies (see Methods).

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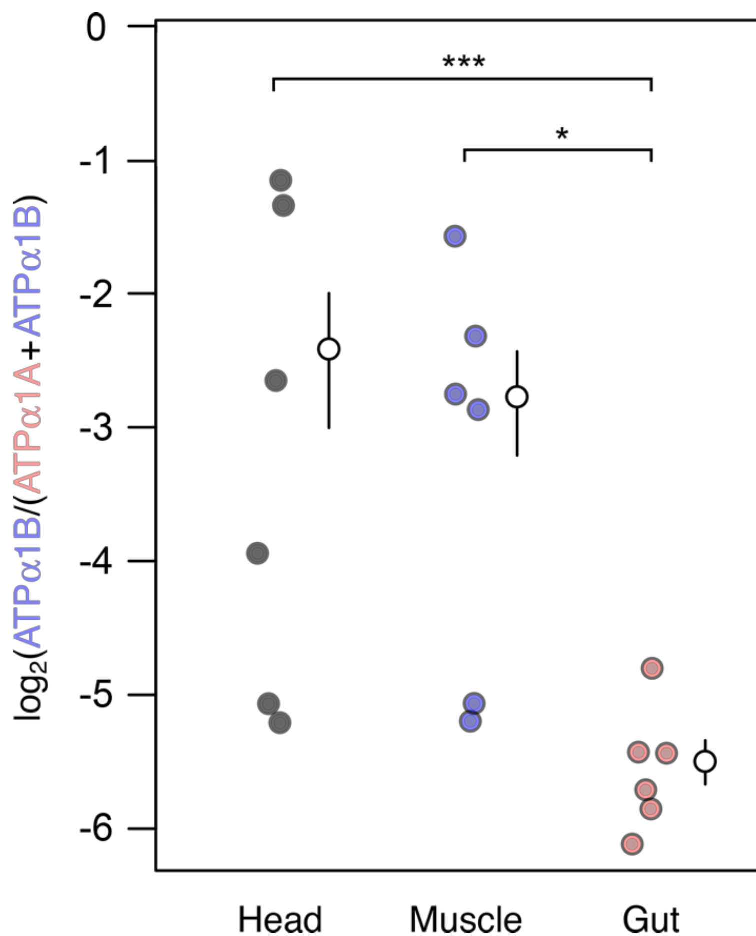

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4 **Figure 4**. RNAseq-based estimates of ATPα1 expression by tissue in the duplication-bearing
5 Orthopteran *Poekilocerus pictus*. As observed in other Apocynaceae-specialists (Zhen *et al*.
6 [29]), the relatively more cardenolide-sensitive ATPα1B is significantly less expressed in the gut
7 than in head (corrected P = 8e-4) and muscle (corrected P = 9e-4) in *P. pictus* (**Table S3**). The
8 mean proportion of ATPα1B of total ATPα1 is indicated with open circles with two standard
9 errors as whiskers. P-values were estimated using the "inverted beta-binomial" test for paired
10 sample count data (see Methods).

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	104	111	112	115	118	119			120	121	122	315	786	797	880	972	
Reference	C	Q	A	V	P	A			D	D	N	I	F	T	R	R	Totals
Z. elegans	·	L	·	·	·	S			·	·	·	·	·	·	·	·	
Largus sp.	·	E	T	·	·	S			·	·	·	·	·	·	-	-	
P. variegata	·	·	·	E	·	N			·	·	·	·	·	·	-	-	
M. latus	·	T	·	E	·	S			·	·	H	·	·	·	-	-	
T. tetraophthalmus	·	L	·	·	·	S			·	·	·	V	·	·	·	·	
L. clivicolis	·	V	·	A	·	N			·	·	H	·	·	·	·	·	
D. subobscura	·	·	·	S	·	S			·	·	·	·	·	·	·	·	
Liriomyza sp.	·	L	·	T	·	S			·	·	·	·	·	·	-	-	
L. asclepiadis	·	·	·	S	·	S			·	·	H	·	·	·	·	·	
N. scrophula	·	L	·	E	·	S			·	·	·	·	·	·	-	-	
P. digitalis	·	L	·	S	·	S			·	·	·	·	·	·	-	-	
D. nerii	·	L	·	·	·	S			·	·	·	·	·	·	·	·	
L. incarnata	·	L	·	·	·	S			·	·	·	L	·	·	-	-	
E. core	·	L	·	·	·	S			·	·	·	·	·	·	·	·	
D. plexippus	·	V	·	·	·	S			·	·	H	·	·	·	·	·	
Parallel		12		2	1	11					5	2					38
Unique		1	1	2													4
P. pictus B	·	L	·	·	·	S			·	·	·	·	·	·	·	·	
P. leprosus B	·	L	·	·	·	S			·	·	·	·	·	·	·	·	
P. pictus A	**·**	**L**	**·**	**T**	**S**	**S**	**A**	**D**	**·**	**·**	**H**	**V**	**·**	**·**	**·**	**·**	
P. leprosus A	**·**	**L**	**·**	**T**	**S**	**S**	**T**	**D**	**E**	**N**	**H**	**V**	**·**	**·**	**·**	**·**	
O. fasciatus D	·	·	·	·	·	S			·	·	H	·	·	·	·	·	
O. fasciatus C	·	·	·	·	·	S			·	·	H	·	·	S	·	·	
O. fasciatus B	**·**	**T**	**S**	**E**	**S**	**S**			**·**	**·**	**H**	**V**	**N**	**·**	**·**	**·**	
O. fasciatus A	**·**	**T**	**S**	**·**	**A**	**S**			**·**	**N**	**H**	**V**	**N**	**A**	**·**	**Q**	
R. lineaticollis A	·	T	·	·	·	S			·	·	·	·	·	·	·	·	
R. lineaticollis B	**Y**	**·**	**·**	**·**	**·**	**S**			**·**	**·**	**Y**	**·**	**·**	**·**	**·**	**·**	
C. auratus A	·	L	·	·	·	S			·	·	·	·	·	·	·	·	
C. auratus B	**·**	**V**	**·**	**·**	**A**	**S**			**·**	**·**	**H**	**L**	**·**	**·**	**S**	**·**	
P. hellebori B	·	·	·	S	·	S			·	·	·	·	·	·	-	-	
P. hellebori A	**·**	**H**	**·**	**S**	**·**	**S**			**·**	**·**	**H**	**·**	**·**	**·**	**-**	**-**	
Parallel		5		3	4					2	3	3					20
Unique	1	1	2				2		1		1		2	2	1	1	14

Figure 5. The pattern of parallel versus unique substitutions at sites implicated in cardenolide-sensitivity with respect to duplication status of ATPα1. Unique substitutions are indicated in red. Relatively less cardenolide-sensitive duplicate copies of ATPα1 are highlighted in bold. Site 874 was excluded from this analysis due to uncertainty in the reconstruction of ancestral states.

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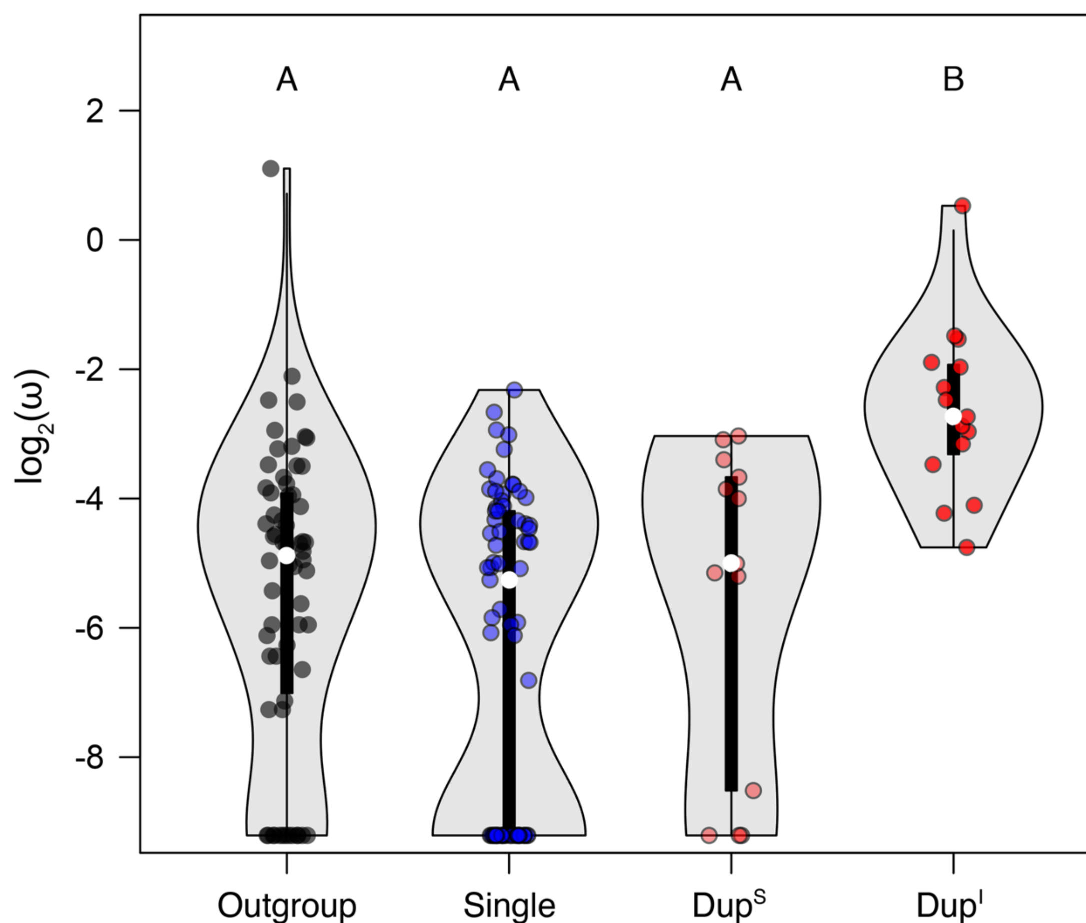

1

2 **Figure 6.** The distributions of omega (dN/dS) estimates for ATPα1 of non-specialists
3 (Outgroup), Apocynaceae-specialists with a single copy of ATPα1 (Single), and specialists with
4 duplicated ATPα1, where the relatively cardenolide sensitive and insensitive copies are noted as
5 Dup^S and Dup^I, respectively. The median omega values are indicated with an open circle and
6 bars represent 50% quantiles. There is a significant difference between the omega distributions
7 for Dup^I and those of all three other groups (Letters A and B indicate significantly different
8 categories; Dup^I vs Outgroup P=2e-5, Dup^I vs Single P=3e-7, Dup^I vs Dup^S P=3e-3). P-values
9 were estimated using Dunn's test of multiple comparisons using rank sums as implement in R
10 (dunn.test) and adjusted using the Benjamini-Hochberg method.

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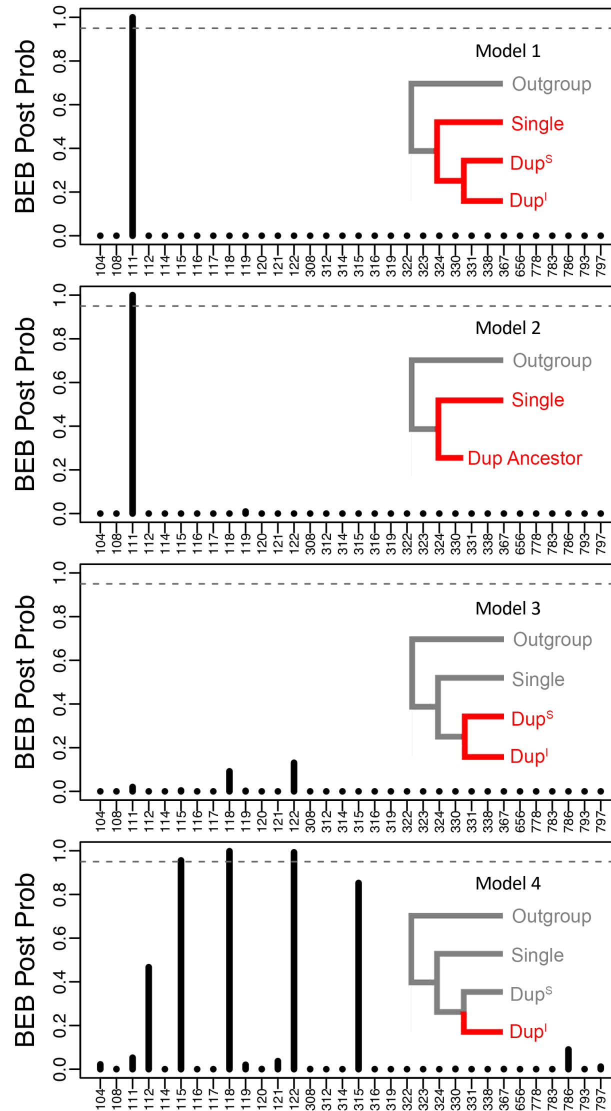

1

2 **Figure 7.** Positively selected sites of ATPα1 among 41 sites implicated in cardenolide-sensitivity

3 (**Table S1**). Sites 802-982 were excluded from the analysis due to missing data. Models 1-4 were

4 tested to identify sites experiencing lineage-specific positive selection. The schematic diagram of

5 each model is shown to the right in each panel. Foreground lineages where positive selection

6 took place are coloured in red and corresponding background lineages are grey. BEB posterior

7 probability >0.95 (grey dashed line) is considered to be strong evidence for positive selection.

8 See **Table S5** for the list of positively selected sites across the whole ATPα1 protein under each

9 model.