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Parallel Molecular Evolution in an Herbivore Community

Ying Zhen^{1,2}, Matthew L. Aardema¹, Edgar M. Medina³, Molly Schumer¹, and Peter Andolfatto^{1,2,*}

¹Department of Ecology and Evolutionary Biology, Princeton University, Princeton, NJ 08544, USA

²The Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544, USA

³Departamento de Ciencias Biológicas, Universidad de los Andes, Bogotá 11001, Colombia

Abstract

Numerous insects have independently evolved the ability to feed on plants that produce toxic secondary compounds called cardenolides and can sequester these compounds for use in their defense. We surveyed the protein target for cardenolides, the alpha subunit of the sodium pump, Na⁺,K⁺-ATPase (ATP α), in 14 species that feed on cardenolide-producing plants and 15 outgroups spanning three insect orders. Despite the large number of potential targets for modulating cardenolide sensitivity, amino acid substitutions associated with host-plant specialization are highly clustered, with many parallel substitutions. Additionally, we document four independent duplications of ATP α with convergent tissue-specific expression patterns. We find that unique substitutions are disproportionately associated with recent duplications relative to parallel substitutions. Together, these findings support the hypothesis that adaptation tends to take evolutionary paths that minimize negative pleiotropy.

Evolutionary genetics has long aimed to understand both the limits of the rate of evolution and the extent to which evolutionary paths are predictable (1). Although it is often presumed that adaptive evolution is mutation-limited (2), others have emphasized the role of pleiotropic and epistatic effects in constraining evolution. In particular, it has been proposed that (i) adaptation may tend to involve regulatory, rather than protein, changes because the former may be less pleiotropic (3); (ii) for similar reasons, specific genes may be hotspots for adaptation (4); (iii) because of epistasis, major evolutionary transitions may be facilitated by small population sizes (4–6); and (iv) that gene duplication may play an important role in the evolution of novelties by releasing constraints imposed by pleiotropy (6–8). The relative importance of each of these factors in organismal evolution is still debated (1, 3, 4, 8, 9). Although there are specific examples for each of these effects, they are often difficult to compare directly because they occur in very different contexts (10–13). Evolutionary parallelisms in settings where different species repeatedly face a common selective pressure provide a natural experiment for addressing these questions, which allows one to assess the extent to which phenotypic changes are caused by the recruitment of a limited set of genes

*To whom correspondence should be addressed. pandolfa@princeton.edu.

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Materials and Methods

Supplementary Text

Figs. S1 to S8

Tables S1 to S11

References (36–80)

and to test whether the specific molecular changes observed involve identical (or highly similar) substitutions (9).

One example of convergence involves the ability of the larvae and adults of a variety of insect species to feed on cardenolide-producing plants of the family Apocynaceae, notably in the genera *Asclepias* (“milkweed”) and *Apocynum* (“dogbane”) (14). Among other antiherbivore adaptations (15), these plants produce a class of secondary metabolites called cardenolides that are toxic to most herbivores. Cardenolides bind to and inhibit the alpha subunit of the sodium pump, Na⁺- and K⁺-exchanging ATPase (Na⁺,K⁺-ATPase) (ATP \square), part of a multisubunit active transporter of Na⁺ and K⁺ that is crucial for muscle contraction, neural function, and other animal cellular processes requiring cation transport. In humans and other mammals, naturally occurring cardenolides regulate the activity of Na⁺,K⁺-ATPase in the context of many physiological processes, and plant-derived cardenolides have been used medicinally for hundreds of years (16). Within insects, herbivores feeding on Apocynaceae include several taxonomic orders spanning 300 million years of insect evolution. Beyond obtaining nutrition from these plants, some of these insects sequester cardenolides for their own defense against predation and have evolved associated aposematic coloration to advertise their unpalatability (17). Perhaps the best-known example is monarch butterflies (*Danaus plexippus*), whose aposematic larvae feed on milkweed plants and sequester large amounts of cardenolide, which in turn provides protection to the larvae and adults from a variety of predators (18).

Insect-host specialization on Apocynaceae plants includes both behavioral and physiological adaptations (15, 19), including amino acid changes in ATP \square . Monarch butterflies carry at least one amino acid substitution (N122H) in ATP \square that confers resistance to the inhibitory effects of cardenolides (20, 21). Additionally, the same substitution (22), as well as a second substitution (L111V) (23), is shared between Danaid butterflies and Chrysomelid beetles, which suggests parallel molecular evolution in these taxa. However, resistant forms of the enzyme in other taxa (e.g., *Rattus norvegicus*, *Hydra vulgaris*, and *Bufo* spp.) lack these specific substitutions; this implies that parallel evolution of cardenolide insensitivity is not always due to the same amino acid substitutions (24–26).

A limitation in examining cardenolide-resistant forms of ATP \square with the exception of Danaid butterflies and Chrysomelid beetles, is that resistance did not arise in the same, or even a similar, ecological context. Additionally, previous work has focused on the 12–amino acid H1–H2 extracellular domain of ATP \square (20–23, 26, 27), yet mutagenesis studies have revealed at least 23 residues scattered throughout the \sim 1000–amino acid ATP \square protein at which amino acid substitutions confer some degree of reduced sensitivity to cardenolides (fig. S1). Structural studies have identified 17 residues that likely directly interact with the cardenolide ouabain or stabilize its binding (28–31), including 12 not detected in mutagenesis studies. Moreover, the states for most residues outside of the H1–H2 extracellular domain have not previously been investigated in cardenolide-sequestering insects (with the exception of the monarch butterfly) (32).

Recurrent gene duplication in Apocynaceae-associated taxa

We investigated the evolution of the ATP \square protein among diverse taxa feeding on Apocynaceae plants, which are subject to similar selective pressures driving the evolution of insensitivity to cardenolides. The genomes of the fruit fly (*Drosophila melanogaster*), pea aphid (*Acyrtosiphon pisum*), monarch butterfly, and flour beetle (*Tribolium castaneum*) contain two, two, three, and four copies of ATP \square respectively, representing two ancient, distinct lineages (which we refer to as ATP \square I and ATP \square II). This duplication likely dates back to the diversification of arthropods (fig. S2). However, the current assembly of the silk

moth (*Bombyx mori*) genome contains just one detectable copy of ATP β that falls in the ATP β clade, which suggests that either the silk moth genome is incomplete or that these ancient duplicates have not persisted in all taxa. In monarch and fruit fly, ATP β genes have relatively low levels of expression (32) (<http://flybase.org/reports/FBgn0040037.html>).

We focused on the evolution of ATP β , the copy common to all of the insects surveyed here, which is evolutionarily constrained at sites implicated in ouabain-binding in outgroup taxa. We performed a survey based on mRNA-Seq transcriptome shotgun sequencing of 26 taxa [including 14 that feed on Apocynaceae (table S1) (33)], as well as an analysis of the complete genomes of pea aphid, silk moth, monarch butterfly, and red flour beetle (33), and we detected single copies of ATP β in all but four taxa. Two copies of ATP β (designated ATP β A and ATP β B) [see (Fig. 1)] were identified in dogbane beetles (*Chrysochus auratus*) and milkweed stem weevils (*Rhyssomatus lineaticollis*), and we detected three copies (designated ATP β A to C) (Fig. 1) in large and small milkweed bugs (*Oncopeltus fasciatus* and *Lygaeus kalmii*, respectively). Our results imply that at least four recent duplications of ATP β have occurred in lineages that feed on Apocynaceae hosts, and none have occurred in lineages that do not [the duplication in the dogbane beetle was overlooked in a previous study (22)]. We estimate that the most ancient of these duplication events, found in large and small milkweed bugs, occurred \sim 60 million years ago (Mya) for copies A and B and \sim 125 Mya for copies C and the ancestor of A and B (33). The estimated divergence time for the gene copies A to C exceeds the divergence between the large and small milkweed bugs (\sim 30 Mya), which implies that all three duplications are likely shared by many species within the Hemipteran subfamily Lygaeidae. The duplications of ATP β observed in the dogbane beetle and the milkweed stem weevil are considerably younger (\sim 24 and \sim 9 Mya, respectively) but may also include multiple species (33).

Accelerated protein evolution in Apocynaceae-associated taxa

A signature of adaptive protein evolution is apparent in patterns of substitution at sites of ATP β implicated in ouabain binding (Fig. 1). Specifically, 33 of the 49 inferred amino acid substitutions occur in lineages that use Apocynaceae (including feeding ability and sequestration of cardenolides) ($P = 1.7 \times 10^{-7}$, accounting for branch lengths sampled) (33). Note that aphids show a distinct pattern (Fisher's exact test, $P = 1.5 \times 10^{-7}$), with all 11 ATP β substitutions having accumulated before the diversification of the oleander aphid and the pea aphid (\sim 65 Mya) (33). If we exclude the aphid-whitefly lineage, the association between amino acid substitutions and use of Apocynaceae therefore becomes stronger, with 33 of 34 substitutions occurring on lineages that use Apocynaceae (Lepidoptera: 5 of 6 substitutions; Coleoptera: 16 of 16 substitutions; Hemiptera: 12 of 12 substitutions; $P = 7.9 \times 10^{-10}$, accounting for branch lengths sampled) (33).

We also see considerable parallelism at the level of amino acid substitution in species that use Apocynaceae. In particular, glutamine at position 111 has experienced 12 substitutions, all of which are observed in at least one other lineage (Fig. 1). Also, position 122 has a replacement of asparagine by histidine in five independent lineages. These observations suggest that sites 111 and 122 are hotspots for substitution in Apocynaceae-feeding lineages (binomial test $P = 6.0 \times 10^{-11}$ and $P = 3.1 \times 10^{-4}$, respectively) (33). Overall, substitutions are significantly clustered in the H1-H2 extracellular domain (i.e., 24 of 33 substitutions occur at positions 111 through 122, binomial test, $P = 9.5 \times 10^{-11}$).

Unique substitutions are associated with duplications

Under a model in which pleiotropic effects constrain evolution, gene duplications may facilitate substitutions that would be deleterious in organisms with a single copy (7, 8). Consistent with this hypothesis, of the substitutions that have occurred on lineages

associated with the use of Apocynaceae, unique substitutions are almost exclusively observed in lineages that carry a recent duplication of ATP α 1 (11:1, $P=0.017$, accounting for branch lengths sampled) (33), whereas parallel substitutions show no such pattern (11:10, $P=0.67$, accounting for branch lengths sampled) (33). Comparing the ratios above confirms that parallel and unique substitutions show significantly different patterns of substitution with respect to duplication status of ATP α 1 (Fisher's exact test, $P=0.027$).

The molecular basis for insensitivity to cardenolides

In a handful of cases (e.g., C104Y, D121N, N122H, Y308C, F786N, and T797A in Fig. 1), we know the effects of particular substitutions on cardenolide-binding affinity from site-directed mutagenesis studies (fig. S1). However, for most substitutions associated with the use of Apocynaceae, we lack this type of detailed information. To examine the likely effects of substitutions in ATP α 1 across insects, we used molecular docking simulations with the crystal structure of the pig ortholog of ATP α 1 bound to the cardenolide ouabain (Fig. 2A) (33). We compared ouabain docking onto the wild-type pig protein with docking onto a protein modified to incorporate individual amino acid substitutions that occurred in lineages associated with use of Apocynaceae (Fig. 1). We show that N122H, which has arisen separately in five lineages and is known from mutagenesis studies to confer resistance to ouabain in cell lines (21), likely acts by sterically blocking ouabain from entering the steroid-binding pocket of ATP α 1 (Fig. 2B). Our docking simulations suggest that four other substitutions (C104Y, N122Y, R880S, and R972Q) have similar effects on ouabain binding (see Figs. 1 and 2C, fig. S3, and table S2). Of these, C104Y is known from functional studies to reduce the affinity of ATP α 1 for ouabain to one-sixth of normal (fig. S1). Thus, although these amino acid substitutions differ, they appear to be functionally parallel. Most other observed substitutions are predicted to have more subtle effects (table S2). For example, the substitution Q111V, which appears in three independent lineages, appears to allow ouabain to fully penetrate the binding pocket (Fig. 2D), although it likely disrupts a key hydrogen bond between the protein and the steroid core of ouabain (Fig. 2A).

Because the ATP α 1 of vertebrates shares only $\sim 77\%$ amino acid identity with insects, it is possible that, in insect lineages, substitutions that ameliorate the ouabain-inhibitory effects of some of the substitutions have arisen elsewhere in the protein. To address this possibility, we performed ouabain-docking simulations on predicted structures for the 19 native proteins of the taxa associated with Apocynaceae (fig. S3) (33). We found a significant positive correlation between the number of substitutions at sites implicated in ouabain-binding (Fig. 1) and the extent of displacement of the best ouabain docking (Spearman rank correlation test, $P=0.007$) (table S3). Further, the 11 native proteins with one or more substitutions predicted to have a large effect (table S2) exhibit significantly greater displacement of ouabain than the eight native proteins lacking such substitutions (Mann-Whitney U test, $P=8 \times 10^{-4}$) (table S3). Finally, we show that the best dockings for ouabain to predicted native protein structures with one or more large effect substitutions are further displaced relative to revertant forms of the native proteins in all 11 cases (Sign test, $P=0.001$) (table S4).

Differential expression of ATP α 1 duplicates

The docking simulations that we performed allow us to predict the extent of ouabain sensitivity of duplicate copies of ATP α 1. Of these duplicates, we expect higher expression of the putatively less sensitive copy in the gut, as this is the primary location of cardenolide processing in these species. In contrast, the copy with the highest level of sensitivity to cardenolides should occur in the brain, because a glial sheath surrounding nervous tissue likely functions as a barrier that prevents cardenolides from reaching Na⁺,K⁺-ATPase in the ventral nerve cord (34). In support of this hypothesis, in the Asian grasshopper *Poekilocerus*

bufonius, which has tissue-specific variation in cardenolide sensitivity, Na⁺,K⁺-ATPase in the brain was found to be the most sensitive to ouabain inhibition compared with Na⁺,K⁺-ATPase of the gut and excretory system (35).

In the dogbane beetle, ATP α B has accumulated five substitutions at sites in the protein that are predicted to determine ouabain-binding affinity (Fig. 1), including N122H and R880S. Docking simulations of ouabain on the predicted structure of native ATP α B suggest significant displacement of ouabain relative to ATP α A (Fig. 3A and table S3) and imply that ATP α B is likely to be less sensitive to ouabain inhibition than ATP α A. These two copies are differentially expressed among tissues (Fig. 3A). The relatively cardenolide-insensitive ATP α B of the dogbane beetle is expressed seven times as much as ATP α A in the digestive tract (Fig. 3A). Conversely, the sensitive ATP α A has three to six times the expression of the less-sensitive ATP α B in the head and thoracic muscle.

Similarly, ATP α B of the milkweed stem weevil harbors two substitutions (C104Y and N122Y) that are predicted to diminish its sensitivity to cardenolides. Our docking simulations imply that this copy of ATP α is also likely to be less sensitive to ouabain inhibition than ATP α A, and the two copies have a pattern of differential expression that is almost identical to that of dogbane beetles (Fig. 3B). We also found the same rank-order of tissue-specific expression of putatively less sensitive to more-sensitive copies of ATP α in large and small milkweed bug, where the sensitivity of the three duplicates of ATP α can be ranked by the total number of substitutions at sites implicated in ouabain-binding and the number of substitutions with large effects identified in docking simulations (table S5 and fig. S4).

Thus, in addition to parallelisms in gene copy number and at the level of individual amino acid substitutions, we also observe parallel evolution of expression patterns for ATP α duplicates arising in distantly related taxa. Differential expression of ATP α duplicates in these taxa may have facilitated the functional specialization of what is otherwise a highly conserved protein.

Discussion

Our results suggest that adaptive evolutionary paths are, at least to some extent, predictable. ATP α is clearly a hotspot for molecular evolution associated with insect adaptation to using Apocynaceae, as seen by repeated duplication of ATP α , parallel changes in gene expression, and parallel amino acid substitutions. The basis for this parallel evolution seems most readily explained by negative pleiotropic effects of most amino acid substitutions in this protein. In particular, we observed that unique substitutions are mostly confined to lineages with duplicate copies of ATP α and that 9 of 11 amino acid substitutions in lineages with one copy occur at only two sites (111 and 122). This level of parallelism is consistent with the hypothesis that negative pleiotropic effects prevent changes at other amino acid sites that could theoretically contribute to reduced sensitivity of ATP α to cardenolides.

Despite the large number of parallelisms observed in the evolution of this system, the pattern in aphids (Fig. 1) indicates that not all substitutions conferring reduced sensitivity to cardenolides at ATP α are necessarily related to adaptation to cardenolide-containing host plants. Notably, the ATP α of aphids is ancestral at many sites implicated in cardenolide binding, contrary to all other insects surveyed here (fig. S5). In addition, ATP α of aphids lacks introns, whereas the fruit fly, flour beetles, and monarchs retain them, which suggests that the ATP α of aphids may represent a retrotransposed copy of the original duplicate. On this basis, we speculate that ATP α and ATP α may perform different roles in aphids than in other insects.

A decade-old debate centers around the relative importance of protein versus regulatory changes and the role of gene duplication in the evolution of novelties [e.g., (3, 8)]. Although our findings point to a certain level of predictability in evolution, they also reveal a plurality of evolutionary responses to a similar selective pressure, which demonstrates how adaptive evolution can leverage a combination of gene duplication, regulatory changes, and protein evolution. The genetic basis of repeated adaptations can be further elucidated by considering contexts where diverse groups of species are faced with a common selective pressure, such as insect-host specialization; cryptic melanism; or environmental gradients in oxygen, salinity, temperature, or acidity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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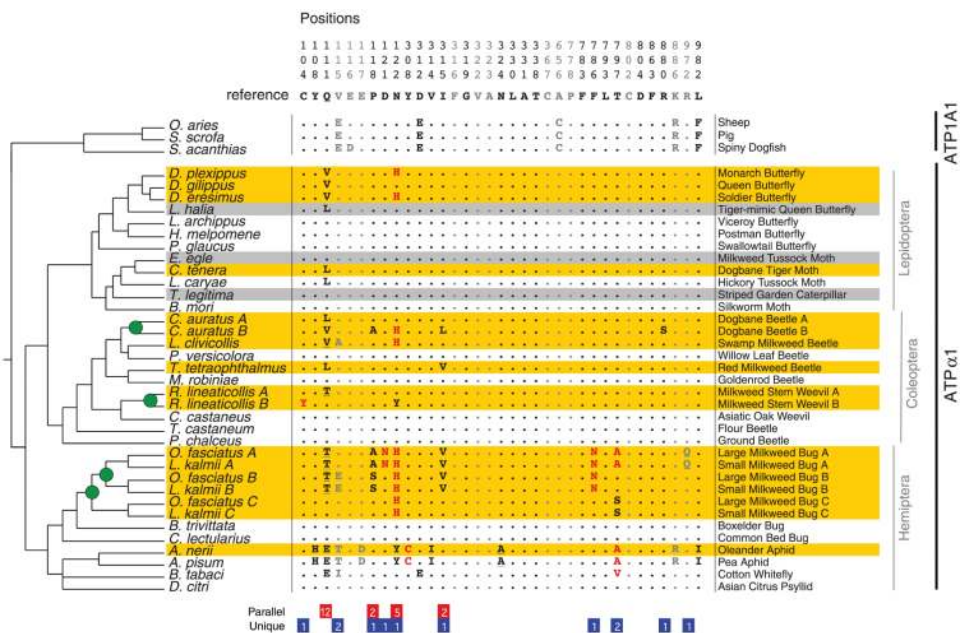


Fig. 1. The phylogenetic pattern of amino acid substitution at sites implicated in cardenolide-binding for ATP $\alpha 1$ in taxa that feed on Apocynaceae and outgroups. Numbered columns correspond to sites for which site-directed mutagenesis or protein structure analysis has suggested a role in cardenolide-binding (fig. S1). Gray sites correspond to those with only structural evidence for a role in cardenolide-binding. Dots represent identity with the consensus sequence. Letters in red are specific substitutions characterized in functional assays (fig. S1). Underlined are substitutions that require at least two nonsynonymous substitutions relative to the ancestral sequence. Orange-shaded rows correspond to specialists known to sequester cardenolides. Gray-shaded rows represent species that either are not known to sequester cardenolides and/or are nonspecialists only occasionally found on Apocynaceae. The cladogram represents the accepted species branching order (table S6), and branch lengths are not meaningful. Green circles represent inferred duplications of ATP $\alpha 1$. Numbers below correspond to the number of inferred substitutions associated with use of Apocynaceae. Red boxes correspond to parallel substitutions (observed in more than one independent lineage). Blue boxes correspond to unique substitutions (observed in only one lineage).

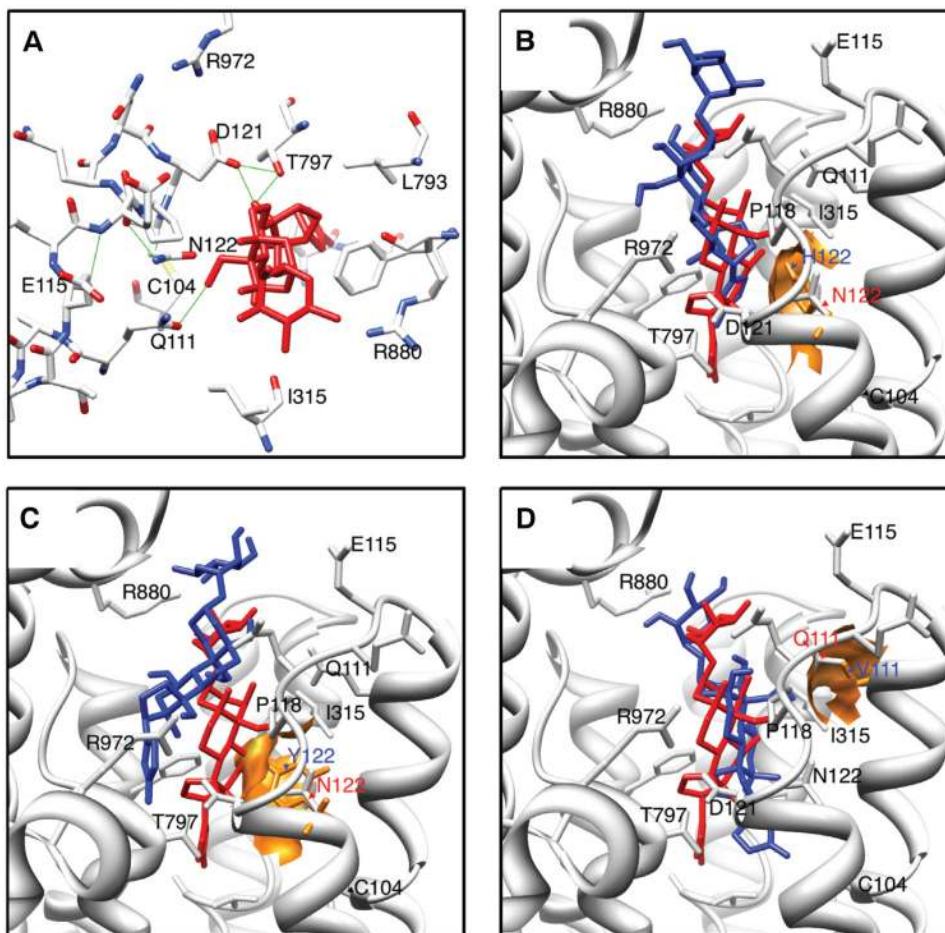


Fig. 2. Cardenolide-binding pocket structure and molecular docking-simulation results. (A) Structure of the cardenolide-binding pocket of ATP1A1 for *Sus scrofa* bound to the cardenolide ouabain (in red) from (31). Green lines represent possible hydrogen bonds between the protein and ouabain. (B to D) Docking simulation results for the substitutions (B) N122H, (C) N122Y, and (D) Q111V. In each case, we show the best docking position for ouabain docked to the native protein (in red) versus the protein with one of three simulated substitutions (in blue). The best docking position is defined as that closest to the coordinates of ouabain in the published cocrystal structure (31). The atomic surfaces of the derived amino acid side chains at position 111 and 122 are detailed in gold. N122H and N122Y sterically block ouabain from entering the binding pocket [root mean square deviations (RMSDs) from cocrystal position are 5.5 and 6.9 Å, respectively; RMSDs from the best wild-type docking position are 5.6 and 6.9 Å, respectively]. Three other substitutions (C104Y, R880S, and R972Q) are predicted to have similar effects (table S2). In contrast, Q111V has more subtle effects on the position of ouabain (RMSD is 2.98 Å from the cocrystal structure and 0.14 Å from the best wild-type docking position) but likely disrupts a stabilizing H-bond (A).

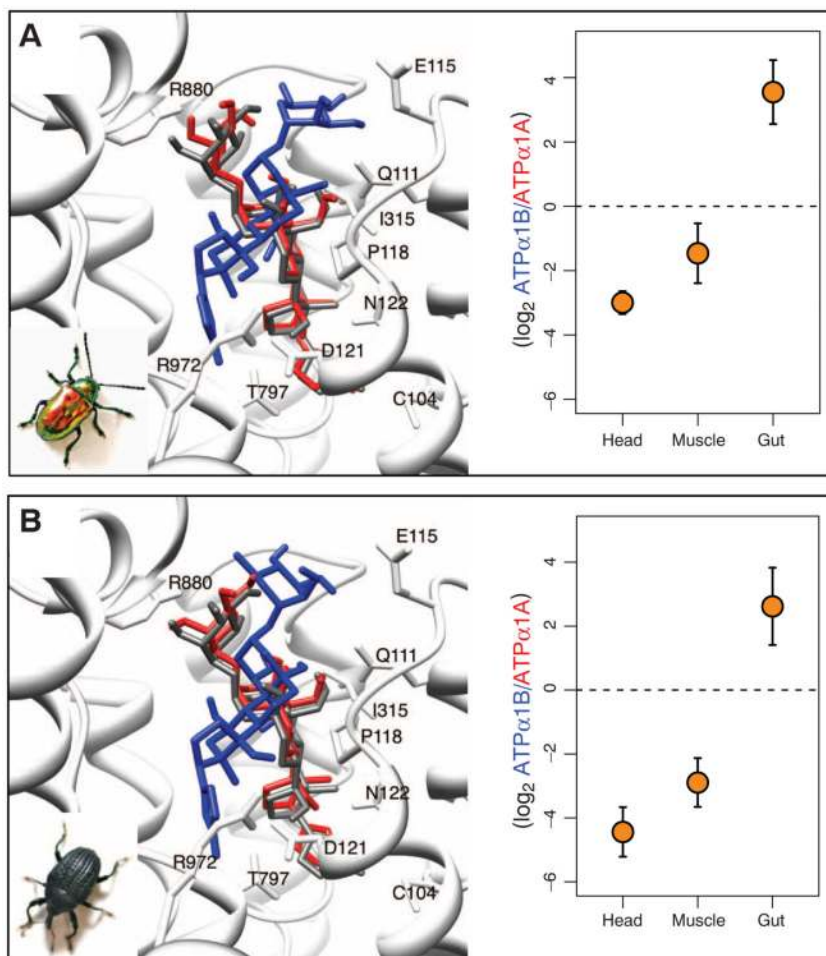


Fig. 3. Native protein–docking simulations and tissue-specific gene expression for ATP α 1B/ATP α 1A duplicates in (A) dogbane beetles and (B) milkweed stem weevils. (Left) Best docking results for ouabain onto pig ATP1A1 (gray ligand) and estimated structures for ATP α 1A (red ligand) and ATP α 1B (blue ligand) for each species (33). For both species, the best dockings of ouabain to the predicted structure of native ATP α 1A are within 1 Å RMSD of the best docking to pig ATP1A1 (table S3). In contrast, the best dockings of ouabain to putatively resistant ATP α 1B copies for dogbane beetles (A) and milkweed stem weevils (B) are displaced by 5.9 and 6.2 Å RMSD, respectively. (Right) Tissue-specific expression means (with two standard errors) of estimates for three unrelated individuals. Positive values indicate higher expression of the putatively resistant copy, ATP α 1B. Analysis of variance (ANOVA) reveals significant variation in expression pattern among tissues (dogbane beetles: $F = 74.3$, $P = 6 \times 10^{-5}$; milkweed stem weevils: $F = 21.1$, $P = 4 \times 10^{-4}$). A Tukey-Kramer test reveals significant differences in all pairwise comparisons except head versus muscle (dogbane beetles, gut-head: $P = 6 \times 10^{-5}$; gut-muscle: $P = 3 \times 10^{-4}$; head-muscle: $P = 0.08$; weevils, gut-head: $P = 4 \times 10^{-4}$; gut-muscle: $P = 3 \times 10^{-3}$; head-muscle: $P = 0.36$).