

Correspondence

# Convergent evolution of cardiac-glycoside resistance in predators and parasites of milkweed herbivores

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The community of plant-feeding insects (herbivores) that specialize on milkweeds (Apocynaceae) form a remarkable example of convergent evolution across levels of biological organization<sup>1</sup>. In response to toxic cardiac glycosides produced by these plants, the monarch butterfly (*Danaus plexippus*) and other specialist herbivores have evolved parallel substitutions in the alpha subunit (ATPA) of the Na<sup>+</sup>/K<sup>+</sup>-ATPase. These substitutions render the pump insensitive to cardiac glycosides<sup>2,3</sup>, allowing the monarch and other specialists, from aphids to beetles, to sequester cardiac glycosides, which in turn provide defense against attacks by enemies from the third trophic level<sup>4</sup>. The evolution of ‘target-site-insensitivity’ substitutions in these herbivores poses a fundamental biological question: have predators and parasitoids that feed on cardiac-glycoside-sequestering insects also evolved Na<sup>+</sup>/K<sup>+</sup>-ATPases that are similarly insensitive to cardiac glycosides (as predicted by Whiteman and Mooney)<sup>5</sup>? In other words, can plant toxins cause evolutionary cascades that reach the third trophic level? Here we show that at least four enemies of the monarch and other milkweed herbivores have indeed evolved amino-acid substitutions associated with target-site insensitivity to cardiac glycosides. These attackers represent four major animal clades, implicating cardiac glycosides as keystone molecules<sup>6</sup> and establishing *ATPalpha*, which encodes ATPA, as a keystone gene with effects that reverberate within ecological communities<sup>7</sup>.

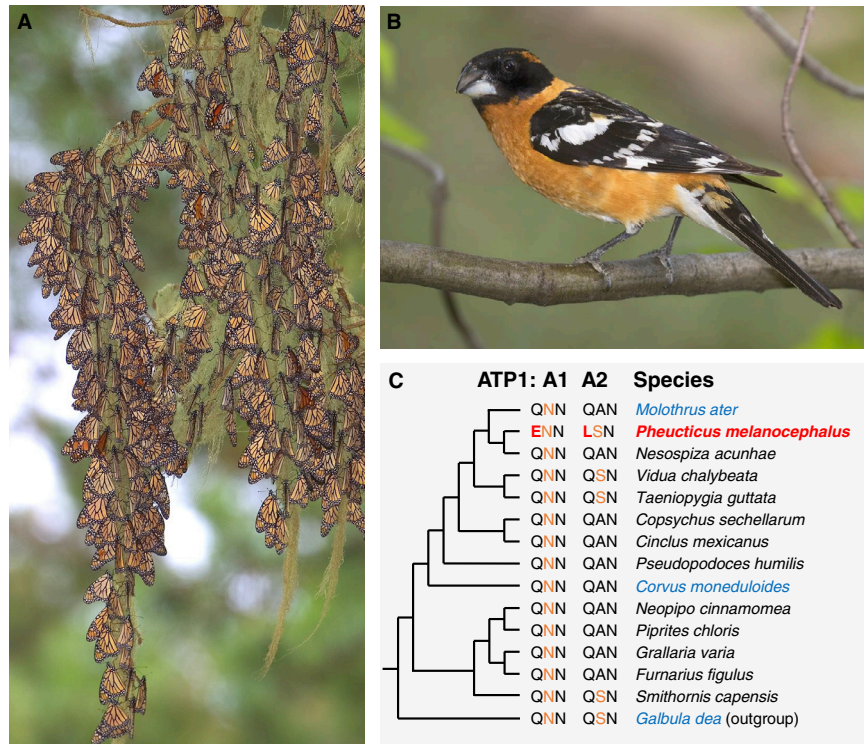
Throughout their range, monarchs serve as abundant, yet dwindling,

prey for a variety of consumers<sup>1</sup>. The black-headed grosbeak (*Pheucticus melanocephalus*), black-backed oriole (*Icterus abeillei*) and black-eared deer mouse (*Peromyscus melanotis*), among other species, are main predators of overwintering adult monarchs in the Oyamel fir forests of central Mexico (Figure 1)<sup>1</sup>. Furthermore, the parasitoid wasp *Trichogramma pretiosum* and entomopathogenic nematode *Steinernema carpocapsae* have been observed or inferred to attack the monarch and/or other cardiac-glycoside-sequestering milkweed specialists<sup>1,8,9</sup>.

We leveraged newly available whole-genome sequences from these and other animal species, coupled with natural history and experimental observations, to reconstruct the occurrence of known and putative

target-site-insensitivity substitutions in the first extracellular loop (M1–M2) of ATPA (Figure 1 and Supplemental information). Avian and mammalian genomes encode four *ATPalpha* paralogs, 1–4. Whereas ATP1A3 and -4 are largely found in nerve or reproductive tissue, ATP1A1 and -2 show high expression in tissues such as muscles (Supplemental information), where Na<sup>+</sup>/K<sup>+</sup>-ATPase function is not protected by the blood–brain barrier (most cardiac glycosides do not penetrate this perineurium, with important exceptions<sup>4</sup>). Therefore, we only selected ATP1A1 and -2 for in-depth analysis.

Among passeriform birds with sufficient genome sequence information available (which did not include the black-backed oriole, *I. abeillei*), we observe that the black-headed



**Figure 1. Natural enemies of milkweed herbivores evolved Na<sup>+</sup>/K<sup>+</sup>-ATPase substitutions known to confer target-site insensitivity to cardiac glycosides.**

(A) Roosting monarch butterflies (*Danaus plexippus*). (B) The black-headed grosbeak (*Pheucticus melanocephalus*) is a passeriform bird predator of the monarch. (C) *Ph. melanocephalus* evolved substitutions that may confer target-site insensitivity in ATP1A1 and ATP1A2, the Na<sup>+</sup>/K<sup>+</sup>-ATPase alpha-subunit (*ATPalpha*) paralogs mainly expressed outside the nervous system (red = may confer target-site insensitivity; orange = may facilitate target site insensitivity; only amino acid positions 111, 119, and 122 are shown). Species in red and blue show resistance or aversion/susceptibility to food items or treatments with cardiac glycosides, respectively; see text for more details. A monarch butterfly roost and black-headed grosbeak were photographed by Dr. Mark Chappell in Point Lobos State Natural Reserve and nearby Big Sur State Park, respectively, in California, USA.



grosbeak (*Ph. melanocephalus*) has substitution Q111E in ATP1A1, which may confer target-site insensitivity<sup>3</sup>. This same substitution evolved convergently in the oleander aphid (*Aphis nerii*) and *Largus* spp.<sup>2,3</sup>, all specialist herbivores of milkweeds (Figure 1). Importantly, Q111E is not observed in ATP1A1 of any other passeriform bird species with available whole-genome sequence data (a further 157 species), and none of these exhibit the extraordinary level of dietary specialization on cardiac-glycoside-sequestering insects as overwintering *Ph. melanocephalus* (Data S1 and Supplemental information). Furthermore, *Ph. melanocephalus* ATP1A2 is unique among known passeriform birds in containing substitutions Q111L and A119S, both of which have been associated with target-site insensitivity (Figure 1). This combination of substitutions evolved numerous times independently in *ATPalpha* paralogs of vertebrate and invertebrate species specialized on diets containing cardiac glycosides<sup>2,10</sup>, and their appearance in *Ph. melanocephalus* coincides with high dietary insensitivity to cardiac glycosides (Supplemental information). Taken together, these observations suggest that these substitutions in ATP1A1 and -2 at least partially belie insensitivity to cardiac glycosides in the grosbeak and facilitate its predation on the monarch, although functional genetic experiments will be required to verify this assertion.

The combination Q111L and A119S also evolved in ATP1A2 of all cricetid and murid rodents we analyzed (Figure S1). Muroid rodents more generally evolved substitution Q111R in ATP1A1 (at the same position as in *Ph. melanocephalus* ATP1A1), which is known to confer cardiac-glycoside insensitivity<sup>10</sup>. Among cricetids, the eastern deer mouse *Pe. maniculatus* is as resistant to cardiac glycosides as another member of the genus, the black-eared deer mouse *Pe. melanotis*, an important predator of the monarch (Supplemental information)<sup>1</sup>. This implies that the target-site-insensitivity substitutions in cricetids facilitate cardiac-glycoside resistance and predation on the monarch as they may do in the grosbeak.

Among invertebrate attackers of the monarch and other milkweed herbivores, we observed that *ATPalpha*

in the genome of the parasitoid wasp *T. pretiosum* encodes substitutions Q111V and A119N (Figure S1 and Supplemental information)<sup>8</sup>. Strikingly, this exact combination of ATPA substitutions at these positions also evolved in the milkweed leaf beetle *Labidomera clivicollis*<sup>2,3</sup>. Substitution A119N may have similar functional consequences as the well-studied A119S, based on several equivalent biochemical properties of the serine and asparagine amino acids<sup>2,3</sup>, whereas Q111V is sufficient for insects to tolerate dietary cardiac glycosides<sup>2,4</sup>.

Entomopathogenic nematodes represent yet another major clade of insect natural enemies. In field studies from central New York, USA on milkweed-herbivore communities, including the cardiac-glycoside-sequestering monarch and red milkweed beetle (*Tetraopes tetropthalmus*), infection was caused exclusively by the native *S. carpocapsae*<sup>9</sup>. Although the *ATPalpha* sequences of all nematode species we analyzed encoded substitutions Q111D and A119S, *S. carpocapsae* also uniquely possessed the substitution N122H, which is known to confer a large target-site-insensitivity effect (Figure S1 and Supplemental information)<sup>2,3</sup>. A119S may facilitate evolution of N122H because the former ameliorates negative pleiotropic effects of the latter<sup>2</sup>, whereas Q111D can further enhance cardiac-glycoside insensitivity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase<sup>3,10</sup>. Future work may establish if these target-site-insensitivity substitutions translate into cardiac-glycoside insensitivity *in vivo* and facilitate a parasitic lifestyle on cardiac-glycoside-sequestering hosts.

In summary, we found that species from four distantly related animal clades that attack milkweed herbivores have each evolved ATPA substitutions that may confer target-site insensitivity to cardiac glycosides<sup>2,3</sup>. The convergent evolution of substitutions conferring target-site insensitivity in ATPA of milkweed-herbivore consumers establishes that plant toxins may bring about a domino effect in which target-site insensitivity substitutions evolve across three or more trophic levels<sup>5</sup>. Our results further highlight the position of cardiac glycosides as keystone molecules and, in turn, *ATPalpha* as a keystone gene<sup>6,7</sup>. Together, cardiac glycosides and a set

of parallel mutations strongly shape patterns of species interactions in the geographically widespread ecosystems that harbor milkweeds.

#### SUPPLEMENTAL INFORMATION

Supplemental information includes one figure, supplemental results and discussion, experimental procedures, supplemental references, acknowledgements and author contributions, and one data file and can be found with this article online at <https://doi.org/10.1016/j.cub.2021.10.025>.

#### DECLARATION OF INTERESTS

The authors declare no competing interests.

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