Title: Defence mitigation by predators of chemically defended prey integrated over the predation cycle and across biological levels.

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Abstract

The long-term evolution of species involved in predator-prey interactions has resulted in many examples of specialised prey defences. The methods that predators use to mitigate prey defences has received less attention. The frequent reference to an arms races or coevolution without clear evidence that both strategies evolved under the influence of each other is problematic. In this review, we use the predation sequence approach as a framework to investigate how predators can evolve traits that allow continued interaction with dangerous prey and we evaluate the evidence for an arms race. We synthesise results from 574 records of predation on prey that are protected by cardiotonic steroids (CTS) – defensive compounds that are found in taxa ranging from toads, to fireflies, to numerous plants, and that have a specific physiological target. We find evidence that distinct lineages of predators share generalised mitigation strategies, and in the latter stages of the predation sequence these strategies are more specific and exploitative behavioural, physiological, and molecular adaptations. In most cases the available evidence does not fulfil the theoretical requirements for arms race dynamics. Our review framework helps to direct future research on what kinds of prey defences appear most profitable for predators to overcome, and what kinds of predatory mitigation strategies are best for a given suite of defences.

Introduction

Predator-prey relationships belong to the most important and well-studied ecological interactions in nature. Prey evolve defences in response to selection from predators, which can be categorised according to the phase of the predation sequence in which they operate [1]. Prey can reduce the chance of *encounter* by avoiding habitats where predators are more common; the chance of *detection* through lack of movement and cryptic appearance [2,3] the risk of *identification* through mimicry or masquerade [4,5]; and the likelihood of being *subjugated* and *consumed* with physical and chemical defences [5,6]. Predators, in turn, develop diverse sensory systems, speed, strength, learning and so on [1]. The interactions between predators and prey have often been regarded as an arms race or a case for coevolution but, in most cases, there is little evidence of co-evolutionary responses by predators.

Coevolution requires a specific reciprocal evolutionary response by both species [7]: new defences by the prey must be continually counteracted by new defence breakers in the predators

and vice versa. Such mutual adaptation is known in host-parasite systems [8], as well as brood parasite-host systems (such as cuckoos, cowbirds, slave-making ants and ant inquilines; [9]). But coevolution in predator-prey systems is comparatively rarer. The specialised antagonistic interactions between toxic newts and garter snakes involving tetrodotoxin is one system where genetic variation for appropriate traits is present, and an evolutionary response by predators show a signature matching of defence-offence that meets the requisites for pair-wise coevolution [10]. While there are a number of cases of specific predator and prey adaptations [11,12], it is often not clear whether both predator and prey evolved under the influence of each other more than one step each, or if some of the adaptations or counter-adaptations result from generalised defence and counter-defence [1].

In this review, we use the predation sequence as a conceptual framework with the aim to understand the types of predator responses to chemically defended prey. This approach has been used successfully for many forms of prey defence and has led to significant insights into the evolution of these adaptations [5,13,14]. This method is particularly useful for predator mitigation strategies as it allows us to bring together a broad range of literatures to form a coherent research field that is better aligned with the broader predator-prey literature. Placing predator strategies into these categories also allows us to investigate whether generalist strategies are more often found in the early stages of the predation sequence, and specialist methods in the later stages, as predicted by Endler over 30 years ago [1].

Just as those before us, who also attempted to bring this literature together [15], we focus our review on a specific interaction between predators and prey: in our case, those that involve cardiotonic steroids (CTS) as a chemical defence. In this system co-evolution is well characterised between specialist insect herbivores and their hostplants [16] but evidence for co-evolution between prey and their predators has received less attention. This system is especially compelling because of the widespread use of CTS as a form of chemical defence across the plant and animal kingdoms, which provides a rich body of comparative data. We start by introducing CTS and their history of research in predator-prey interactions; then briefly review the different prey animals that are defended by CTS and the predators that feed on them, before delving into the different methods that predators use to mitigate CTS defences across the predation sequence.

We integrate these methods across biological levels of organisation, from biochemistry, to physiology, to microbiology, and to behaviour. We discuss the costs and benefits of attacking CTS-defended prey because this is integral for our understanding of the fitness consequences and selective pressure on predators and the ecological dynamics of predator-prey interactions. Our aim is to promote research that encompasses more integrative investigations of the diverse and multi-faceted mechanisms influencing the evolution of this system, and to suggest where researchers can focus their studies to shed light on whether a coevolutionary arms race is ongoing between predators and prey.

1. A brief introduction to and history of CTS in predator-prey evolution

CTS are a diverse group of compounds derived from triterpenoids that are found primarily in plants, but also in animals (figure 1; [16]) and have a specific physiological target, the transmembrane protein Na^+, K^+−ATPase (NKA, [17,18]). CTS are found in prey organisms on every continent, and cardenolide diversity and concentration are variable among prey species and individuals [19]. There are two classes of CTS: cardenolides and bufadienolides. Both are produced *de novo* in plants and animals [19], and some animals also sequester CTS from their host plants or prey [20,21]. This sequestration has almost certainly evolved as a defence against predators [22–24]. CTS are toxic because they bind to the extracellular surface of the transmembrane protein Na^+, K^+−ATPase (NKA, [17,18]) and, when bound, disable passage of Na^+ and K^+ across the membrane. This disrupts electrochemical gradients causing many physiological systems to become dysregulated [25]. Although the NKA is highly conserved among animals, independent evolution of NKA insensitivity to cardenolides has occurred in six taxonomic orders of insects that specialise on cardenolide containing plants.

In many cases CTS consumption results in predators rejecting prey and learning to avoid them [23], which, in over 40+ years of research, was decoded by Brower and colleagues [20,24,26,27]. Focusing on the monarch butterfly (*Danaus plexippus*) that as caterpillars feed on milkweed plants (*Asclepias*) and sequester cardenolides [16], Brower and colleagues revealed the chemical and pharmacological basis of the butterfly's chemical defence [28,29]. When *Asclepias*-fed monarchs were presented to blue jays (*Cyanocitta cristatata*) the birds consumed them and universally responded by vomiting, and subsequently avoided attacking the monarchs in future

encounters [24]. Brower and his colleagues also pioneered research on resistant predators, providing the first evidence for species of birds and rodents that were immune to the toxic effects of CTS [24,30–32]. They were the first to hypothesise that resistant predators had likely undergone changes to their gustatory systems, and that physiological resistance evolved in the ancestors of bird and rodent predators of monarchs – topics that we cover in sections 4.2 and 5.2, respectively [33,34]. But, 30 years on, the role of NKA in CTS resistance of these bird and rodent predators have not been functionally studied, although predicted resistance-conferring genetic substitutions have been identified [35].

2. Taxonomic distribution and diversity of CTS in prey

The two main classes of CTS compounds – cardenolides and bufadienolides – differ in the structure of the steroid backbone and lactone group (the aglycone; figure 1). Cardenolides, are primarily produced in plants and comprise a steroid backbone structure with a five-membered lactone group and a sugar moiety attached to C-3 of the first carbon ring [16]. The subset of CTS that possess a sugar moiety on C-3 are known as cardiac glycosides because their side chains are derived from sugars (are glycosylated). Bufadienolides have a six-membered lactone ring at C-17 and typically lack a sugar moiety [16]. Despite its frequent use in the literature, the term "cardiac glycoside" does not cover the majority of bufadienolides found in animals, which are non-glycosylated. For this reason, we use the umbrella term cardiotonic steroid, except for the cases where we can refer to specific CTS class.

Sequestration of dietary cardenolides is known from members of several Lepidoptera families, including Danaidae [36] and Arctiidae [37–40]. The sequestered cardenolide profile in monarch butterflies is dependent on host plant characteristics and larval developmental stage [27,41,42]. Several beetles synthesize their own cardenolides [43,44], and cardenolides have also been detected in Eurasian toads, *Bufotes viridis* [45] and African crested rats, *Lophiomys imhausi* [46]). Bufadienolides are most often found in toads (family *Bufonidae*) (reviewed in [47]), and the bufadienolide profiles from skin secretions of toads vary significantly from species to species, and even within species by population [48–53]. Lucibufagins – a subclass of bufadienolides – are believed to be synthesized from cholesterol by fireflies (mainly from the subfamily *Lampyrinae*). Species of the genus *Photuris*, which are members of the sister group to

Lampyrinae, cannot synthesize their own lucibufagins and instead acquire them by preying on lucibufagin-producing fireflies [54–57]. Lucibufagins are also sequestered by keelback snakes of the genus *Rhabdophis* in a remarkable example of a dietary shift from eating toads to eating fireflies [58]. Other animals that are chemically defended by CTS include a wide range of insects that mostly sequester cardenolides from their plant hosts. Sequestering insects include beetles of the cerambycid genus *Tetraopes* and chrysomelid genus *Chrysochus* [59–61]; as well as some aphids (Homoptera: Aphididae, oleander aphid, *Aphis nerii;* [62]); bugs (Heteroptera: Lygaeidae (*Oncopeltus fasciatus* and *Lygaeus kalmi*; [59,63]), and grasshoppers (Orthoptera: Pyrgomorphidae [64]). Finally, several beetles are known to synthesize their own cardenolides. These include the chrysomelids of the genera *Oreina* [43] and *Chrysolina* [44], which use bright and conspicuous colouration to signal their chemical defences to predators, otherwise known as aposematism [4].

3. Taxonomic distribution of predators of CTS-defended prey

We searched published records of predators feeding on CTS-defended prey using search strings in Google Scholar and the natural history notes from herpetological reviews. Search strings included one or more of the following terms: toad, *Bufo*, bufonidae, milkweed, *Danaus*, monarch, fireflies, diet, cardiac glycoside, cardenolide, bufadienolide, Lampyridae, predation and predator names accumulated during the search. As taxonomic designations have changed repeatedly, especially among bufonidae 'true toads', it was also necessary to work backwards and forwards from review articles and field guides which had citations using previous versions of species names. Only the current species names, reconciled from GBIF, were used for the final list.

Our database (supplementary table 1) includes 574 records of predation of CTS-defended prey. The evidence comes from field observations as well as feeding studies with captive animals. 73% of the reports related to the predation of toads, while the rest documented predators that feed on non-toad CTS-defended prey (lepidoptera, fireflies, grasshoppers, true bugs, beetles, and aphids). Both anurans and caudates consume toads of one or more life stage, and toad-eating is widespread among snakes (see [65] for a review). Entire genera either feed exclusively on toads or make toads a crucial part of their diet (e.g., hognose snakes (*Heterodon spp.* [66–70]),

keelbacks (*Rhabdophis spp.*) [58,71,72]; night adders (*Causus spp.*) [73,74]; garter snakes (*Thamnophis spp*.) [75–77], and xenodontines (*Xenodon spp*.) [78,79]). Toad eating is also observed in mammals (mustelids and rodents [80–82]), some shorebirds, waterbirds and waterfowl, and aquatic invertebrates that typically feed on eggs, hatchlings and tadpoles [83] (see figure 2). One of the most remarkable predators of toads are the nymphs of some epomis beetles [84–86] which capture juvenile toads with an elaborate luring strategy (see section 4.1 on encounter [87]).

Over thirty vertebrate species are known to eat monarch butterflies with minimal adverse effects (supplementary table 1). And arthropod predators include lacewings, ants, spiders, ladybirds, cockroaches, mantids, predatory stink bugs, assassin bugs, and wasps [88–93]. The most striking example of bird predators that have succeeded in breaking through the cardenolide defence of the monarch are the mixed- and single-species flocks of birds including the black-headed grosbeak (*Pheucticus melanocephulus*) and the black-headed oriole (*Oriolus larvatus*), which kill an average of 15,000 butterflies per day in the large overwintering aggregations in Mexico [30,94]. Species of mice that are found near monarch overwintering aggregations (including *Peromyscus aztecus*, *Reithrodontomys sumichrasti*, *Neotomodon alstoni*, and *Microtus mexicanus*) also feed heavily on the butterflies. An individual *P. melanotis* can consume an average of 37 monarchs each night [31]. Over the winter season, the mice account for ~5% of the total predation on the monarch colony (a population of *P. melanotis* can attack 100-3000 monarchs per night [31]). Paper wasps can also kill and eat up to 5000 monarch caterpillar larvae [95], and their choice of monarchs varies depending on the species of milkweed on which the larvae have fed.

A number of vertebrates are known to eat the other main CTS-defended insects – fireflies [96]. Bats have been observed chasing firefly adults, but surprisingly only big brown bats (*Eptesicus fuscus subsp. Fuscus*) have been confirmed to have fireflies in their diet [97]. Likewise, anoles such as *Anolis evermanni* and *A. cristatellus* have been suggested to be avid consumers of fireflies while the likelihood of other anoles eating fireflies depends on their level of satiation [98]. The worm-eating clade of keelback snakes, which includes *Rhabdophis nuchalis* and *R. leonardi*, have shifted their regular diet of earthworms to occasionally include firefly larvae [58].

Doing so allows them to sequester lucibufagins from the fireflies for use in their own chemical defence (see section 6.1).

Generalist predators made up 84% of our records while 6% could be considered as specialists (including birds, insects, mammals, and reptiles). We found that behavioural adaptations were more often reported in generalists than specialists, and that molecular resistance (confirmed by functional assay) is present in both generalists and specialists, but has been tested in only 5% of the predators known to eat CTS defended prey. For the majority of specialists, whether their degree of CTS tolerance matches prey-specific defensive chemistry remains untested.

4. How do predators overcome CTS defences?

In the following sections we uncover the potential evolutionary relationships between CTS specific defences and predator adaptations at the different stages of predation. Our intention is not to provide an exhaustive list of all mitigation strategies, but to provide the reader with an idea of the diversity and parallelism of these strategies, and a simple way in which they can be categorised.

4.1 Encounter

The first stage of predation is for predators to situate themselves such that they increase their chances of encountering CTS-defended prey. Prey abundances and distributions change over time and space, which creates a complex changeable environment [99]. The life history and demographics of different predators can increase the probability of their encountering CTS-defended prey. For example, the common frogs (*Rana temporaria*) breed earlier and their offspring develop faster than natterjack toads (*Epidalea calamita*), which allows common frog tadpoles to eat toad spawn and newly hatched tadpoles, and results in 100% toad mortality [100]. Predators also move to areas where CTS prey are found, such as the adult *Peromyscus melanotis*, which migrate in large numbers to areas where monarch butterflies aggregate in the winter. *P. melanotis* feed on monarchs and breed successfully, whereas four other species of mice do not breed because they are deterred by the monarchs' defences. Predators can also lure prey during encounters, as seen in trophic role reversal by larvae of ground beetles (genus *Epomis*; [87]).

Larvae of *E. circumscriptus* and *E. dejeani* move their antennae and mandibles in the presence of frogs and toads, which triggers amphibian predation behaviour. The larvae avoid the predator's attack by ignoring toe wagging by the amphibians, and instead attach to the amphibian's body and start feeding.

4.2 Detection, identification, and approach

After finding potential prey, predators must detect and decide whether the prey are worth attacking. Deciding to approach CTS-defended prey requires a predator to overcome the initial reluctance that most naïve individuals express after encountering CTS-defended prey [101,102]. This can be facilitated and maintained via intergenerational cultural transfer [103], i.e., foraging by older individuals who consume chemically defended prey without ill effects can locally enhance foraging by younger less experienced predators (i.e., optimal action is to shift to attacking the prey [104] [105]). Social transmission of prey approach and handling has been suggested for black-headed grosbeaks (*Pheucticus melanocephalus*) that feed on monarch butterflies [24], and by Torresian crows (*Corvus orru*) that feed exclusively on the nontoxic parts of toads [106]. Socially acquired prey preferences can also be modified later in life [107]. For example, fringe-lipped bats (*Trachops cirrhosis*) acquire a novel association between the call of a toad species and palatable prey after observing the positive foraging experience of a conspecific [108].. This type of reversal learning is important when thinking about the identification and fitness of edible auto-mimics (e.g., monarch butterflies that lack cardenolides) because if predators acquire enhanced identification of prey profitability through social transmission, this should influence how frequency-dependent selection operates on prey [109]. Because social transmission of avoidance is beneficial for defended prey [109] we would expect selection to favour prey to evolve traits that maximize opportunities for social learning about identification such as new, perhaps more salient, multimodal defences [110] that increase distastefulness to elicit strong disgust responses [111]. The three systems (grosbeaks, crows, and bats) present compelling opportunities to test the role of social information of different populations of predators' attack decisions (identification stage) and capture (approach stage) and the potential for reciprocal responses by prey.

4.3 Subjugation

Once predators have approached prey they must handle and subdue them. We found that dissecting behaviour is a common trait in predators (figure 2), including insects [112–114], mammals and birds [106,115,116], and even in limbless predators such as snakes [117]. At first glance, dissecting behaviour is a surprising evolutionary solution for snakes. However, it is made possible because of the enlarged posterior maxillary teeth [117] which are thought to have evolved to allow deep tooth penetration into prey, as well as for other non-predatory purposes such as male-male combat [118]. Dissecting behaviour is innate in some mustelids [80,119,120], and in some birds this behaviour is thought to be exapted from fruit-eating, and would therefore be of low cost to maintain given its benefit in other contexts [24,94]. Dissecting behaviour may evolve and be maintained via cultural transfer [106] because headshaking in response to aversive stimuli could be used by conspecifics to guide dissecting behaviour [121], and for individuals to develop discriminatory chemosensory behaviour [24].

The widespread occurrence of dissecting behaviour suggests a shared ability to taste and avoid CTS in predators [122]. Although cardenolides are often described as bitter tasting compounds [123], we lack comparative tests on the chemosensory detectability of CTS. Japanese tiger keelback snakes (*Rhabdophis tigrinus*) show no discrimination between purified bufadienolides and control stimuli [124], which suggests that there are other chemosensory signals that the snakes use during predation. On the other hand, single cardenolides do elicit taste discrimination by birds and this varies with cardenolide polarity [125]. In adult monarch butterflies, cardenolides are nearly twice as concentrated in the wings than the rest of the body and are especially concentrated in the wing-scales, which gives predators that attack this part of the body a mouthful of bitter compound [38]. Whether this is an evolutionary response to predation, and whether predators that attack monarchs vary in their ability to detect and tolerate cardenolides in a manner that matches the concentration in the wings is yet to be systematically investigated but could be evidence of differential co-evolution.

Some predators, such as *Peromyscus melanotis*, and European hedgehogs (*Erinaceus europaeus*), which feed on CTS-defended prey, have significantly higher taste rejection thresholds for single cardenolides, monarch butterflies, and cardenolide-defended grasshoppers (*Poekilocerus bufonius*) compared with other closely related species that do not feed on CTS-

defended prey [126]. There appears to be sufficient intraspecific variability in this behaviour to have resulted from natural selection but this is yet to be investigated [127]. Taste insensitivity to cardenolides suggests that either the taste receptor genes have undergone functional changes, or that the valence of CTS have changed, or can be changed, from negative to positive. Future research comparing the g-protein coupled Tas2r taste receptors responsible for bitter taste perception could reveal patterns of evolution related to prey defences and predator diet [128,129].

4.4 Consumption

Evolved avoidance of CTS by dissecting or eating the least CTS-laden parts of prey is one possible result of predator-prey interactions, but does not necessarily represent the kind of escalating counteradaptation to prey defences expected for coevolutionary arms races. If an arms race-type process is occurring, we expect matched levels of CTS defence of prey and resistance ability of the predator [15]. In this section we describe target-site insensitivity (TSI) via amino acid substitutions in the CTS binding pocket of the NKA and its potential as a candidate for predator-prey coevolution.

Most vertebrates possess three paralogs of the NKA subunit α gene (ATP1A1-3) that have tissue-specific expression profiles and are associated with distinct physiological roles. Most amino acid variation among species and paralogs is concentrated in the H1-H2 extracellular loop (residues 111-122), which shows clade- and paralog-specific patterns of variability but also show remarkable patterns of convergence, parallelism, and divergence [130]. Amino acid substitutions at sites 111 and 122 in particular have been found to be key in the evolution of TSI in insect and vertebrate species [131] and have evolved in snakes [65,132], frogs [133,134], and other vertebrates [130].

Many birds that are sympatric with invasive toads, but have no evolutionary history of co-occurring with toads, have no amino acid substitutions likely to confer resistance [135]. Snakes that have shifted their diet from eating toads to eating fireflies do have TSI [58]. It has been hypothesised that the black headed grosbeak which feed on monarch butterflies also possess amino acid substitutions in two of the three paralogues which is likely to confer resistance

[136,137]. In other species of birds that are reported as specialist feeders of CTS-defended danaid butterflies [138], such as bulbuls (*Pycnonotus barbatus*) and hornbills (*Lophoceros eucomelas*), genome annotations of ATP1A1 of related species also show potential TSI-conferring substitutions in both ATP1A1 and -A2. In other predators, such as the generalist egg parasitoid wasp, *Trichogramma pretiosum*, and in the generalist entomopathogenic nematode *Steinernema carpocapsae*, potential TSI-subsitutions are also present [137].

Understanding the evolutionary history and potential for co-evolution of a trait requires some knowledge of the patterns of variation among individuals, populations, and species [15]. Where functional tests of TSI substitutions have been performed, there can be greater than 10-fold variation in TSI among enzymes that have identical paired states at 111 and 122 [130], as well as significant variation in enzyme activity, which together suggest that substitutions at other sites also contribute to CTS resistance through intramolecular epistasis and can be subject to selection [137,139]. Enzyme function, however, is but a proxy for predicting effects on organismal fitness, and research exploring how the effects of adaptive mutations at the protein level cascade to the whole-organism fitness, and how they match the defences of prey in different populations and locations will be necessary to understand the potential for coevolution.

5. Mitigation strategies after consumption yet to be explored

If CTS-consuming animals do not use TSI to avoid intoxication, how do they survive? Insect and vertebrate CTS can vary ontogenetically [27,41,42] from species to species and within populations [48–53], in terms of concentration, diversity [16], and polarity, which can influence their chemosensory detectability [125], toxicity [140], transport [141], and excretion [142]. CTS also vary seasonally and geographically [143,144], which may influence selection for TSI. In this section we draw on the information from plant-herbivore interactions where insects that that possess sensitive NKA still feed on cardenolide-defended plants [40,145,146]. We discuss how predators could possess guts that are impermeable to cardenolides via biological barriers [147,148]; how hormonal systems can mitigate loss of NKA activity; and the scope for gut microbiota to neutralise the toxicity of CTS.

5.1 ABC transporters and binding proteins

One method to avoid CTS-toxicity which has not been explored in predators is having an impermeable barrier to non-polar CTS [149,150]. Polar and hydrophilic CTS are unable to passively cross the gut and perineurium due to epithelial diffusion barriers such as septate junctions, and thus pass through predator bodies without causing toxicity [141]. But for non-polar CTS, the presence of P-glycoprotein efflux carriers, which are well-known for their function in maintaining the blood–brain barrier of animals and have been identified in gut epithelial cells, could increase resistance to the toxins. Indeed, mice with P-glycoprotein deficiencies (mdr1a gene knockouts) respond with increased CTS levels in their tissues (particularly in the brain) after intravenous injections of the toxins compared to wildtype mice [151].

Binding proteins could also contribute to CTS-resistance in predators [152]. Binding proteins typically transport non-polar steroid hormones through the bloodstream to their target cells, where in some cases interactions with docking proteins cause them to release the steroids [17,153]. Because endogenous CTS function in regulating cardiac contractility and circulation [25], it is possible that a binding protein system for transporting CTS to specific targets such as cardiomyocytes is already in place. Previous studies have shown that mammals possess a CTS-specific binding protein, which binds to the steroids with high affinity and inhibits their function [154,155]. These binding proteins are produced at high concentrations in the kidneys, where they likely protect the NKA of those tissues [153]. Gene sequences for these proteins, however, are still lacking and we do not know whether such a mechanism could provide substantial protection to a predator that ingests high concentrations of CTS.

5.2 RAAS and the enlargement of adrenal glands

A particularly interesting morphological pattern that has been identified in snakes that feed heavily on toads is extreme adrenal gland enlargement [156], which suggests that the renin-angiotensin-aldosterone system (RAAS) could play a role in mitigating CTS toxicity. Increased physiological stress from processing CTS could lead to higher production of stress hormones (i.e., corticosteroids and catecholamines) that results in adrenal enlargement. However, hormonal responses to bufadienolides in *Rhabdophus tigrinus*, show no increase in circulating corticosteroid levels in response to bufadienolide injections [157]. Alternatively, increased

production of the mineralocorticoid hormone aldosterone in the enlarged adrenal glands could compensate for reduction in NKA activity caused by CTS by increasing NKA expression (figure 4) [158,159]. Increased circulating aldosterone has been identified in the Japanese toad-eating snake *R. tigrinus* [157], which exhibits highly enlarged adrenal glands [156]. Furthermore, garter snakes (*Thamnophis elegans*) injected with bufadienolides responded with significantly increased NKA expression in their heart tissue [160]. However, whether CTS exposure directly leads to increased circulating aldosterone and NKA expression, and consequently adrenal gland enlargement in resistant predators requires further experimental tests.

5.3 Gut microbiota

Gut microbiota are known to neutralise the toxicity of CTS by metabolising CTS to reduced/inactivate compounds such as digoxin to dihydrodigoxin [161]. The bacterial source of digoxin metabolism has been traced to the Actinobacterium *Eggerthella lenta*, and the mechanism is linked to a multi-gene operon known as the cgr (cardiac glycoside reductase) [161,162]. In the presence of digoxin cgr genes are significantly upregulated, allowing *E. lenta* to inactivate digoxin by reducing its lactone ring (i.e., dihydrodigoxin). This modification is believed to distort the ring planarity leading to reduced binding to NKA. The cluster of genes that make up the cgr operon include eight genes, which are present in individuals that can metabolise digoxin and are absent in non-metabolizers, thus representing a single genetic locus predictive of digoxin metabolism. Functional tests of one of the eight genes, Cgr2, alone show that it is sufficient for digoxin inactivation [163] and that it has strict specificity for cardenolides (e.g., digoxin, ouabain, ouabagenin, digoxigenin, digitoxin). How widespread gut bacteria that can digest CTS are in predators, and whether they are a key step to a predator's adaption to CTS-defended prey remains an open question.

6. Benefits and costs of consuming CTS

Having covered the range of known and potential predator mitigation strategies we now discuss the costs and benefits of these strategies. This is necessary if we are to draw conclusions about the selective pressure on predators and therefore the ecology and evolution of these strategies. Many of the examples we have described in section 4 and 5 could be applied to any chemical defence mitigation, and likewise many of the benefits could also apply broadly. For example

coping with toxic prey can expand predator niches by providing a competitive release [164] as seems to be the case with the population of scansorial black-eared mice (*Peromyscus melanotis*) that are larger, heavier, and reproduce more than mice of the same species whose territories are outside of the overwintering monarch roosts [31]. Thus, in this section, we cover the specific aspects of predator counteradaptations to CTS and propose a range of putative benefits and costs, and suggest how these could be measured.

6.1 Defence against a predator's own enemies

Some predators sequester CTS from their diet for redeployment in their own chemical defence. Hedgehogs self-anoint skin secretions from toads onto their spines [165], as do African crested rats (*Lophiomys imhausi*), whose hairs are highly specialized to wick up and store the cardenolide that they chew from the roots and bark of *Acokanthera schimperi* (Apocynaceae) [46]. When threatened during approach, these two very different species have evolved similar behaviours and warning displays: African crested rats part the hairs along their flank to reveal both warning coloration and their poison-laced hairs, and Japanese tiger keelback snakes (*Rhabdophis tigrinus*) which store bufadenolides in specialized nuchal glands on the back of their necks [21] arch their necks towards the threat revealing brightly coloured yellow and red skin covering the nuchal glands [166]. In some cases, pressure created by the arching of the neck breaks the skin, causing the stored toxins to shoot out towards the attacker (experienced personally by SM). Japanese tiger keelback snakes also maternally provision bufadenolides to their offspring via embryonic transfer. Female snakes have been found to actively forage for toads during gestation, when they are depositing yolk into their ova [167], and the amount of CTS in the nuchal glands of offspring corresponds proportionally to the amount found in the mother [168]. The few members of *Rhabdophis* that have shifted their diets away from frogs and toads to smaller invertebrate prey occasionally feed on CTS-defended firefly larvae to maintain the defence benefit provided by CTS sequestration [58]. Whether other predators such as black headed grosbeaks use cardenolides for defence without active sequestration mechanisms, as has been found for other organisms that tolerate toxin consumption [169], is an open question. This is possible, given that other species of grosbeak appear to have toxins in their feathers [170], and their orange and black colour could give them a transient defensive advantage against their own predators.

Many species of bufophagous (i.e., toad-eating) snakes also death feign in response to an attack [66,171–173]. The behaviour is not exclusive to bufophagous snakes, and at least one species of highly bufophagous snake (*Causus rhombeatus*) does not feign death [174]. However, the enlargement of the adrenal glands in several species of bufophagous snakes [156] is thought to be linked to this behaviour. Increased catecholamine production by enlarged adrenal glands could lead to a parasympathetic syndrome preceding death feigning [174]. Because CTS may render toad-eating snakes distasteful, "death-feigning" may slow a predator's attack and increase the predator's detection of CTS [175].

Beyond chemical defence sequestration, predators may take advantage of CTS to protect themselves from parasites (reviewed in [176]). "Self-medication" [177] has not been investigated in predators that feed on CTS, but the diverse pharmacological properties of these compounds suggests that such an evolutionary relationship is possible. For example, several bufadienolides have been shown to have antimicrobial and antifungal properties [53,178].

6.2 Behavioural, physiological and molecular costs

As generally expected for adaptations, CTS resistance comes with a cost, but the evidence for this is scarce and indirect. Dissecting behaviour and slower prey handling may translate into an overall cost in fitness in some species [95]. Otters, for example, can ingest frogs immediately but require more time to skin, wash, and select the parts to ingest from a toad [115]. Black headed grosbeaks (*Pheuticus melanocephalus*) and orioles (*Icterus parisorum*) that feed on monarch roosts feed on a 7.85 day on-off cycle [103], and also change their feeding depending on ambient temperature [179], which is likely due to the changes in toxicity with ambient temperature [180,181]. Shifts in feeding patterns probably reduce the impact of cardenolide toxicity but increase opportunity costs of foraging over short windows of time. Whether this behaviour is evidence for detoxification costs or is a cost of TSI requires further study. In mice, introducing resistance-conferring substitutions that occur in wildtype ATP1A1 onto ATP1A2 negatively affects their learning ability, locomotor activity, and anxiety-related behaviours [182]. A similar trade-off has also been observed in Australian snakes that feed on toads, which show reduced

performance, locomotor capability, and increased prey handling time compared to non-toad eaters [183,184].

Endowing a protein with a new function through mutation often incurs a cost, particularly with respect to the protein's original function [130,134,146,185]. Functional studies of TSI have repeatedly shown that resistance-conferring substitutions often carry substantial functional costs to the ATPase activity of NKA [130,134]. These negative pleiotropic effects can have major implications at higher biological levels due to the vital role that NKA have in the maintenance of physiological homeostasis. Animals that have evolved TSI through substitutions at sites 111 and 122 have thus either co-adapted additional substitutions that compensate for such negative pleiotropic effect [134,146,185] or, as is the case with neotropical grass frogs of the genus *Leptodactylus* (Leptodactylidae) that feed on toads (but do not specialise on them), undergone a tandem duplication of ATP1A1 and subsequent neofunctionalisation of one copy, which allows them to maintain a highly resistant and a highly functional versions of the protein [134,186].

7. A broader view of CTS resistance in predators and the coevolution with CTS-defences in prey

In this review we have drawn together the evidence about the methods that predators use to overcome the suite of defences deployed by CTS-defended prey. We have shown that dissecting behaviour is used by invertebrates, reptiles, birds, and mammals; that changes in perception of risk and of taste perception has occurred in mammals and birds; and that target-site insensitivity (TSI) via amino acid substitutions in the CTS binding pocket of the NKA has evolved in parallel in invertebrate and invertebrate predators. We have also pointed to biochemical, hormonal, and microbiological strategies that have yet to be investigated in this context. In all cases, however, tight coevolution [7] remains an elusive conclusion in this predator-prey system. Why is this, when there is evidence of co-evolution in another system of toxic prey and predators [187]?

Using the predation sequence as a framework, it becomes apparent that variation in the consequence of the interaction between a predator and prey influences the strength of selection on defence mitigation strategies by predators (see also [15]). In the interactions between the garter snake *Thamnophis sirtalis* and the rough-skinned newt *Taricha granulosa,* tetrodotoxin

(TTX; [188]) is a potent neurotoxin that blocks voltage-gated sodium channels in nerve and muscle tissue, and inhibits the propagation of action potentials. Newts show individual variability in TTX quantity, just as CTS-defended prey show ontogenetic and individual variability in toxin concentration and diversity [189]. But the difference between the two systems is that sensitive predators cannot survive the ingestion of newts, either because it kills them directly or incapacitates them, rendering them susceptible to predation and reducing their ability to thermoregulate. Whereas, we have shown that predators of CTS-defended prey can interact with them without necessarily suffering the lethal consequences of the toxin. This difference in the selective pressure between the two systems also explains a pattern that has emerged during our synthesis of the data, which is a tendency for many generalised predation methods to have evolved (optimal foraging, social learning, dissecting behaviour, changes in gustatory perception). There are three potential reasons for this. (1) Generalised methods may be less expensive than specialised methods because they are used continuously and for other purposes such as finding mates and holding territories, or are evolutionary responses to the predator's own predators or competitors [12]. For example, detecting and identifying CTS-defended prey is based on general sensory and cognitive properties such as diverse sensory systems, learning ability, and primarily fit within optimal foraging theory [190]. In many cases predators choose prey on the basis of their overall availability and profitability [191][192]. (2) Because prey defences that operate early in the sequence are generalised and only generalised methods are required to overcome them, but in the later stages of the sequence prey defences are more specific and the risk to predators increases, with predators "forced" into experiencing selection (also proposed by Brodie III and Brodie Jr. [15]). Finally, (3) the interactions between predators and CTS-defended prey are more diffuse than between snakes and newts due to the community complexity of these natural predator-prey systems. Our analysis shows that most predators prey on several species, and therefore the total selective pressure on each other is more diffuse, making it more challenging to detect co-evolutionary dynamics between any one pair of species.

Understanding the evolutionary history and potential for co-evolution of a trait requires some knowledge of the patterns of variation among individuals, populations, and species. This is well known for CTS-defended prey, but is still generally lacking for predators. Our review has highlighted potential areas to explore in predators: chemosensory perception, TSI, toxin-binding

proteins, and gut microbiota. This research field will benefit from more detailed within- and between-population analyses of these traits to quantify individual variation, which is necessary for selection to act. In many cases it appears that predators are pre-adapted to feeding on CTS, i.e., muroid rodent TSI. Reconstructions of the evolutionary history of predators and co-occurrence with CTS prey, and their dietary specialisation on – or tolerance to – CTS-defended prey will be important for understanding whether these animals are pre-adapted to attack CTS-defended prey [33,193] or whether TSI evolved directly from exposure to CTS, and whether there is evidence for ongoing co-evolution.

8. Conclusions and future directions

Understanding the full range of mechanisms contributing to toxin resistance in predators of toxic prey is an important goal for evolutionary biology. The recurring emergence of predators that can feed on and exploit CTS-defended prey has involved remarkable convergence in the behaviours, physiology, and molecular mechanisms by which they achieve this adaptation. Although a majority of research focus has revolved around target-site insensitivity of NKAs, we have found that there are multiple physiological, chemosensory, behavioural, and ecological mechanisms that can also contribute to, and consequently shape, this adaptation. In Table 1, we list key questions that could be addressed in our continued quest to understand the mechanisms that have shaped this adaptation.

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Author contributions:

S.M.: Conceptualisation, investigation, writing – original draft, visualisation, supervision, and project administration; L.Y.: Software, formal analysis, resources, data curation, writing – review and editing, visualisation; M.B.: Investigation, data curation, writing – review and

editing. H.M.R.: Conceptualisation, investigation, writing – original draft, visualisation, supervision and project administration, funding acquisition.

560 **Table 1.** List of open questions for future studies aiming to expand our understanding of the
561 mechanisms of CTS-resistance in predators of toxic prey.
562

	Question	Experimental scheme(s) to address question
Chemosensory	How do the taste receptor genes of CTS-resistant predators compare to those of sensitive predators?	• Comparing the Tas2r genes of *Peromyscus* species that have varying sensitivity to cardenolides compared to related species of mice would reveal the underlying molecular mechanisms of CTS tolerance.
	Can predators that dissect chemically identify CTS-laden tissue?	• Modifying either real or artificial CTS-defended prey so that the CTSs are stored in different parts of the body and observing the dissecting behaviour of predators would reveal whether they consistently avoid the same part of the body or whether they can detect CTS and avoid whichever part of the body contains it.
Molecular mechanisms of resistance	Are ABC transporters protecting additional tissues in predators of CTS-defended prey?	• P-glycoprotein transmembrane proteins are encoded by the ABC (ATP-binding cassette) transporter gene superfamily [194]. The genes encoding these proteins fall into seven subfamilies (A-G) and have ancient eukaryotic origins [195]. ABCG2 or ABCG2-like genes have been found in 41 bird species, and ABCG2-like genes have been lost in only five species [196]. We recommend sequencing the ABC transporters and comparing expression patterns in resistant and non-resistant predators to determine whether these proteins are upregulated to protect important tissues. It is possible to express ABC transporters in cell culture to assay their ability to bind to relevant CTS [197] and such studies would confirm their ability to protect tissues. Exploring the co-evolution of ABC and ATP1A genes in predators will be a key step in understanding the stages of evolution of CTS resistance.
	Are binding proteins helping to protect tissues from CTSs?	• Isolating binding proteins from plasma and sequencing amino acids would help identify the gene(s) encoding these proteins. • Measuring plasma levels of these binding proteins in resistant vs. nonresistant predators would reveal whether they play an adaptive role in predators of CTS-defended prey.

Physiological mechanisms of resistance	Does the renin-angiotensin-aldosterone system (RAAS) play a role in CTS resistance?	• Rearing hatchling CTS-resistant animals (snakes or mice) on a diet with and without CTSs and then monitoring circulating aldosterone levels on a long-term basis, followed by comparing adrenal gland morphology and tissue-specific NKA expression levels would reveal if and how the RAAS system adapts to a CTS-heavy diet.
	Are there physiological costs to resistance?	• Investigating the effects of amino acid substitutions in ATP1A genes in vitro and in vivo with CRISPR-Cas9 would reveal how pleiotropic effects at the protein level cascade to the whole-organism level. This could subsequently reveal what physiological systems might be co-adapted with target-site insensitivity.
	Are there physiological costs to feeding on CTSs?	• Comparing the physiology and performance of CTS-resistant predators fed CTS-defended prey (toads) vs. control prey (non-toad frogs) would reveal whether digesting the compounds is physiologically demanding and provide insights into the cost of this adaptation.
Role of gut microbiota	How widespread are gut bacteria that can digest CTSs and are they key to a predator's adaption to CTS-defended prey?	• Comparing CTS metabolizing ability of stool cultures from predators of CTS-defended prey and those that avoid them would reveal whether there are CTS-metabolizing bacteria in the guts of predators. • Comparing the composition of the microbiota between predators of CTS-defended prey and those that avoid such prey would reveal potential CTS-metabolizing strains. • Inoculating germ-free resistant and nonresistant predators with CTS-metabolizing strains would reveal whether gut microbes can augment resistance or confer resistance on their own.
	Are there *cgr* genes in the gut microbiome of cardenolide-feeding animals?	• Because *cgr* genes were found to be responsible for the ability of some bacteria to metabolize cardenolides, a screen for these genes in the microbiomes of resistant and nonresistant species could point to whether gut microbiota contribute to CTS resistance in predators of CTS-defended prey.

Behaviour	Are some CTS-feeding animals self-medicating against parasites?	• The Japanese tiger keelback snake (*Rhabdophis tigrinus*) is known to have high and highly variable parasite loads [198,199]. These snakes feed on toads and sequester bufadienolides into specialized nuchal glands on the back of their necks. The amount of bufadienolide in their nuchal glands directly correlates with the number of toads they have ingested. Measuring their bufadienolide contents and parasite loads would reveal whether they correlate with one another.

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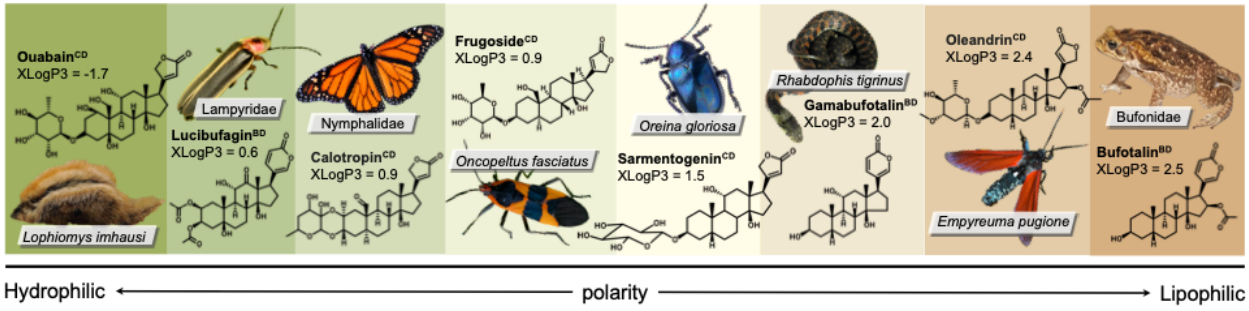

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565

566 **Figure 1.** Axis of polarity of CTS produced or sequestered by animals. CTS polarity is
567 represented by octanol-water partition coefficients (predicted by XLogP3). This is not an
568 exhaustive list of CTS found in each prey source, but illustrates key characteristic compounds.
569 Cardenolides (denoted by ^{CD}), which are generally glycosylated, tend to have higher polarities
570 than bufadienolides (denoted by ^{BD}), which are not glycosylated. Polarity data were obtained
571 from the National Center for Biotechnology Information's PubChem. Photo credits: crested rat
572 (*Lophiomys imhausi*) by Don McCulley (2018); firefly (*Photinus sp.*) by Katja Schulz (2018);
573 monarch butterfly (*Danaus plexippus*) by Peter Miller (2014); milkweed bug (*Oncopeltus*
574 *fasciatus*) by Judy Gallagher (2017); cobalt milkweed beetle (*Chrysochus cobaltinus*) by Oregon
575 Department of Agriculture (2016); tiger keelback snake (*Rhabdophis tigrinus*) by Yasunori Koid
576 (2009); spotted oleander wasp moth (*Empyreuma affinis*) by Shaina Noggle (2010); cane toad
577 (*Rhinella marina*) by Brian Gratwicke (2012).

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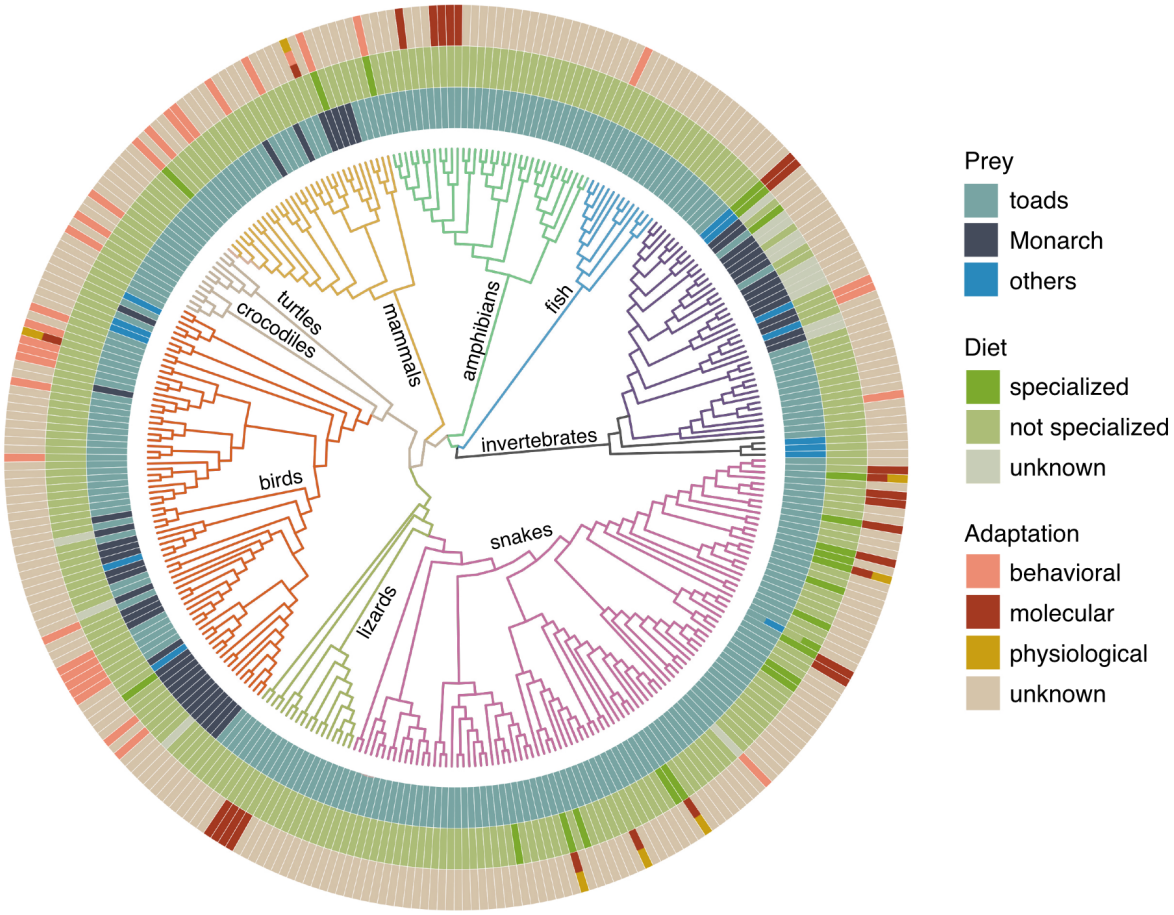

Figure 2. Phylogenetic tree of predators of CTS-defended animals including true toads (Bufonidae spp.) and milkweed butterflies (*Danaus spp.*), including monarchs. Information on behavioural, molecular, and physiological adaptation is scarce and unevenly reported for different animal groups. Only those confirmed by functional experiments are marked as having molecular resistance to CTS. Phylogenetic relationships were inferred from timetree.org. References for prey, diet, and adaptation characterizations are available in supplementary table 1.

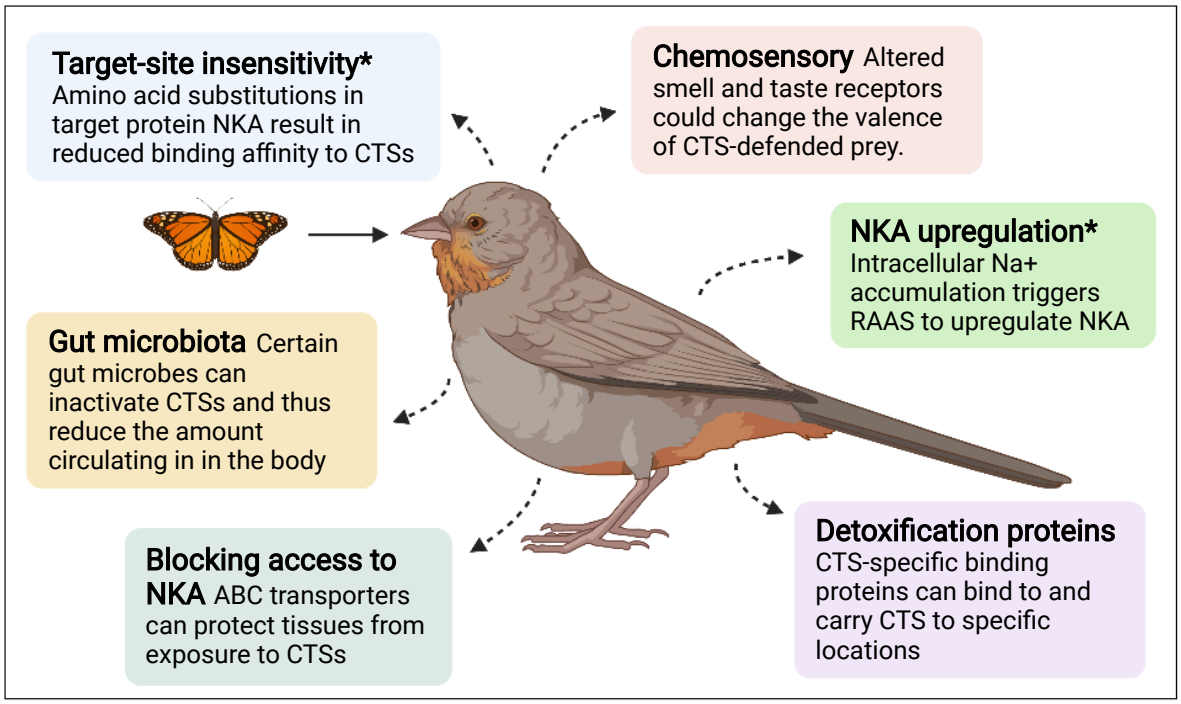

Figure 3. Summary of different potential mechanisms that can contribute to resistance in predators of CTS-defended prey. Mechanisms that have been empirically linked to contributing to a predator's ability to overcome CTS toxicity of defended prey are marked by an asterisk. Predators may avoid feeding on prey parts with high concentrations of CTS or detoxify CTS after ingestion. In addition, they may possess altered target sites that are no longer susceptible to the toxic action of CTS. Some predators sequester CTS from their prey and defend themselves against their own predators (e.g., snakes of the genus *Rhabdophis*). Less attention has been paid to metabolic transformations that allow predators to detoxify CTS and excrete the resulting metabolites. These diverse mechanisms can influence a predator's behaviour, which in turn influences ecological interactions, and ecological structures. Figure created with BioRender.com

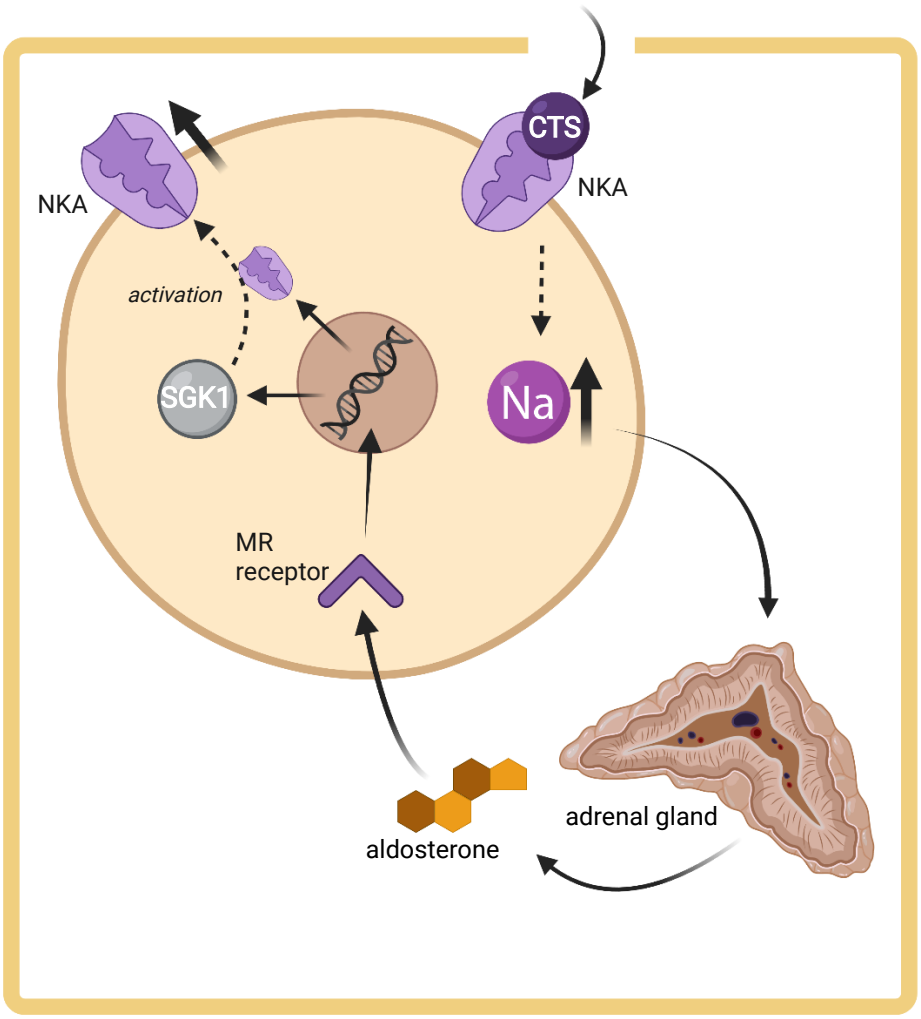

Figure 4. A schematic diagram of how the adrenal glands can signal the expression of NKAs following CTS exposure. CTS enters the organism, reaches a cell, and disables NKAs, causing an increase in intracellular Na+ because the disabled proteins no longer transport Na+ out of the cell. This triggers the adrenal glands to secrete the mineralocorticoid hormone aldosterone, which passes through the cell membrane and binds to an intracellular mineralocorticoid (MR) receptor. This receptor translocates into the nucleus where it activates a transcriptional program inducing expression of modulators of sodium transport such as SGK1 and also NKAs themselves. Figure created with BioRender.com and based on data from [200,201].

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