Review

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Defence mitigation by predators of chemically defended prey integrated over the predation sequence and across biological levels with a focus on cardiotonic steroids

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Predator–prey interactions have long served as models for the investigation of adaptation and fitness in natural environments. Anti-predator defences such as mimicry and camouflage provide some of the best examples of evolution. Predators, in turn, have evolved sensory systems, cognitive abilities and physiological resistance to prey defences. In contrast to prey defences which have been reviewed extensively, the evolution of predator counter-strategies has received less attention. To gain a comprehensive view of how prey defences can influence the evolution of predator counter-strategies, it is essential to investigate how and when selection can operate. In this review we evaluate how predators overcome prey defences during (i) encounter, (ii) detection, (iii) identification, (iv) approach, (v) subjugation, and (vi) consumption. We focus on prey that are protected by cardiotonic steroids (CTS)—defensive compounds that are found in a wide range of taxa, and that have a specific physiological target. In this system, coevolution is well characterized between specialist insect herbivores and their host plants but evidence for coevolution between CTS-defended prey and their predators has received less attention. Using the

predation sequence framework, we organize 574 studies reporting predators overcoming CTS defences, integrate these counter-strategies across biological levels of organization, and discuss the costs and benefits of attacking CTS-defended prey. We show that distinct lineages of predators have evolved dissecting behaviour, changes in perception of risk and of taste perception, and target-site insensitivity. We draw attention to biochemical, hormonal and microbiological strategies that have yet to be investigated as predator counter-adaptations to CTS defences. We show that the predation sequence framework will be useful for organizing future studies of chemically mediated systems and coevolution.
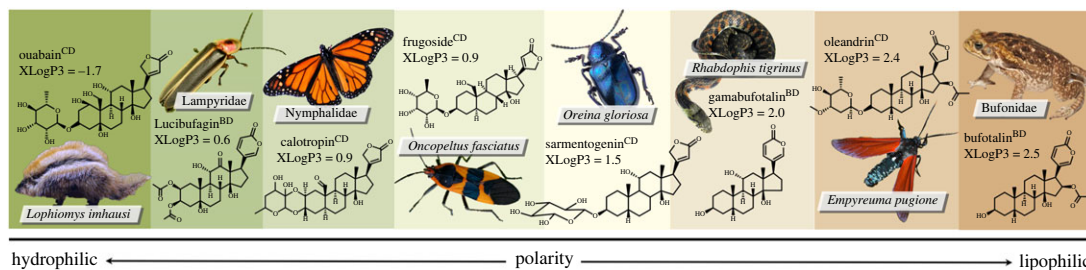
1. Introduction

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 categorized 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 an example of coevolution but, in most cases, there is little evidence of coevolutionary responses by predators.

In this review, we use the predation sequence as a conceptual framework with the aim to understand the types of predator strategies that evolve in response to attacking 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 [5,7,8]. 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 generalized predation methods, which we define as methods that apply to many different types of prey, are more often found in the early stages of the predation sequence, and specialized methods—those that are more prey-specific—in the later stages. This was predicted by Endler over 30 years ago [1].

Just as those before us, who also attempted to bring together the literature on the evolution of predators in response to chemically defended [9], 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, coevolution is well characterized between specialist insect herbivores and their host plants [10], but evidence for coevolution 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 organization, 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.

2. A brief introduction to and history of cardiotonic steroids 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; [10]) and have a specific physiological target, the transmembrane protein Na$^+$, K$^+$-ATPase (NKA, [11,12]). CTS are found in prey organisms on every continent, and their diversity and

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

concentration are variable among prey species and individuals [13]. There are two classes of CTS: cardenolides and bufadienolides. Both are produced de novo in plants and animals [13], and some animals also sequester CTS from their host plants or prey [14,15]. This sequestration has almost certainly evolved as a defence against predators [16–19]. CTS are toxic because they bind to the extracellular surface of the transmembrane protein NKA [11,12] and, when bound, disable passage of Na^+ and K^+ across the membrane. This disrupts electrochemical gradients causing many physiological systems to become dysregulated [20]. Although the NKA is highly conserved among animals, independent evolution of NKA insensitivity to cardenolides has occurred in six taxonomic orders of insects that specialize on cardenolide containing plants [21].

In many cases, CTS consumption results in predators rejecting prey and learning to avoid them [16], which, in 40+ years of research, was decoded by Brower and co-workers [14,17,22,23]. Focusing on the monarch butterfly (*Danaus plexippus*) that as caterpillars feed on milkweed plants (*Asclepias*) and sequester cardenolides [10], Brower and co-workers revealed the chemical and pharmacological basis of the butterfly's chemical defence [24,25]. 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 [17]. Brower and co-workers 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 [17,26–28]. They were the first to hypothesize that resistant predators had probably 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 §§5.2 and 6.2, respectively [29,30]. 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 [31].

3. Taxonomic distribution and diversity of cardiotonic steroids 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 [10]. 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 [10]. 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 [32] and Arctiidae [33–36]. The sequestered cardenolide profile in monarch

butterflies is dependent on host plant characteristics and larval developmental stage [23,37,38]. Several beetles synthesize their own cardenolides [39,40], and cardenolides have also been detected in Eurasian toads, *Bufotes viridis* [41] and African crested rats, *Lophiomys imhausi* [42]. Bufadienolides are most often found in toads (family Bufonidae) (reviewed in [43]), and the bufadienolide profiles from skin secretions of toads vary significantly from species to species, and even within species by population [44–49]. While CTS are found in the parotoid glands of adults, they have also been detected at lower concentrations in ovaries, oocytes, eggs, tadpoles, plasma and bile [43]. 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 [50–53]. 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 [54]. 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* [55–57]; as well as some aphids (Homoptera: Aphididae, oleander aphid, *Aphis nerii* [58]), bugs (Heteroptera: Lygaeidae (*Oncopeltus fasciatus* and *Lygaeus kalmi* [55,59]) and grasshoppers (Orthoptera: Pyrgomorphidae [60]). Finally, several beetles are known to synthesize their own cardenolides. These include the chrysomelids of the genera *Oreina* [39] and *Chrysolina* [40], which use bright and conspicuous coloration to signal their chemical defences to predators, otherwise known as aposematism [5,61].
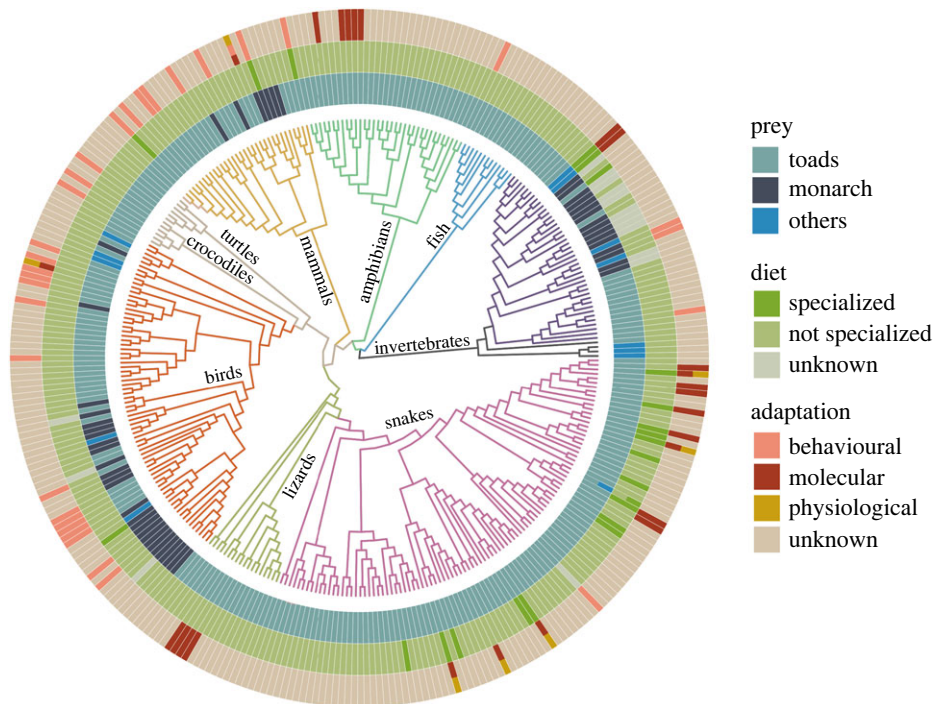
4. Taxonomic distribution of predators of cardiotonic steroids-defended prey

There are two main ways of classifying predators. One is to use a 'trophic classification' method: carnivores consume animals, herbivores consume plants, and omnivores consume prey from more than one trophic level. Our preferred alternative is a 'functional' classification of the type outlined in [62]: true predators, grazers, parasitoids and parasites. Using this definition, we searched Google Scholar for published records of predators feeding on CTS-defended prey. We also searched the natural history notes from herpetological reviews. We used the search strings that included the prey animals known to contain CTS defences and the types of CTS from section two with the word predator or predation. Our search strings included: Danaidae, Arctiidae, *Danaus*, monarch butterfly, Eurasian toad, toad, *Bufo*, Bufonidae, cane toad, *Bufotes viridis*, African crested rat, *Lophiomys imhausi*, fireflies, Lampyrinae, *Photuris*, snake, keelback snake, *Rhabdophis* cerambycid, *Tetraopes*, chrysomelid, *Chrysochus*, *Chrysolina Oreina* Homoptera, Aphididae, oleander aphid, *Aphis nerii,* Lygaeidae, milkweed bug, *Oncopeltus fasciatusi, Lygaeus kalmi*, grasshoppers, Orthoptera and Pyrgomorphidae. 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 the Global Biodiversity Information Facility (GBIF), were used for the final list.

After reviewing the titles and abstracts of the search results, we had a final dataset of 574 records of predation of CTS-defended prey (electronic supplementary material, table S1). These records include field observations as well as feeding studies with captive animals. Seventy three per cent 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 [63] 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. [64–68]), keelbacks (*Rhabdophis* spp.) [54,69,70]; night adders (*Causus* spp.) [71,72]; garter snakes (*Thamnophis* spp.) [73–75] and South American hognose snakes (*Xenodon* spp.) [76,77]). Toad eating is also observed in mammals (mustelids and rodents [78–80]), some shorebirds, waterbirds and waterfowl, and aquatic invertebrates that typically feed on eggs, hatchlings and tadpoles [81] (figure 2). One of the most remarkable predators of toads is the nymph of some epomis beetles [82–84], which captures juvenile toads with an elaborate luring strategy (see §5.1 on encounter [85]).

Over 30 vertebrate species are known to eat monarch butterflies with minimal adverse effects (electronic supplementary material, table S1); arthropod predators include lacewings, ants, spiders, ladybirds, cockroaches, mantids, predatory stink bugs, assassin bugs and wasps [86–91]. The most

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 electronic supplementary material, table S1. Figure made with the phytools package in R.

striking example of bird predators that have succeeded in breaking through the cardenolide defence of the monarch is 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 [26,92]. 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 deer mouse (*Peromyscus melanotis*) can consume an average of 37 monarchs each night [27]. Over the winter season, the mice account for approximately 5% of the total predation on the monarch colony (a population of *P. melanotis* can attack 100–3000 monarchs per night [27]. Paper wasps can also kill and eat substantial numbers of monarch larvae, in both controlled indoor experiments and in the wild where they fly from their nests to forage on plants containing larvae (up to 5000 monarch caterpillar larvae were killed and eaten over the course of one study by Rayor [93]). The paper wasp's choice of monarchs also varies depending on the species of milkweed on which the larvae have fed [93].

A number of vertebrates are known to eat the other main CTS-defended insects—fireflies [94]. 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 [95]. 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 [96]. 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 [54]. Doing so allows them to sequester lucibufagins from the fireflies for use in their own chemical defence (see §5.1).

We classified predators as generalist if they tend to exploit a wide variety of resources, although they might exploit one if it is very abundant, and specialists if they are species adapted to exploit a single food type or niche, but will exploit other niches either opportunistically or when primary food is in short supply [97]. From this we found that 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.

5. How do predators overcome cardiotonic steroids 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 categorized.

5.1. 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 [98]. 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 can result in 100% toad mortality [99]. Predators also move to areas where CTS prey are found. Adult *P. melanotis* migrate in large numbers to areas where monarch butterflies aggregate in the winter and feed on monarchs and breed successfully, whereas four other species of mice do not breed because they are deterred by the monarchs' defences [30]. Predators can also lure prey during encounters, as seen in trophic role reversal by larvae of ground beetles (genus *Epomis*; [85]). 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.

5.2. Detection, identification and approach

After encountering 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 naive individuals express after encountering CTS-defended prey [100,101]. This can be facilitated and maintained via intergenerational cultural transfer [102], 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 [103,104]). Social transmission of prey approach and handling has been suggested for black-headed grosbeaks (*Pheucticus melanocephalus*) that feed on monarch butterflies [17], and by Torresian crows (*Corvus orru*) that feed exclusively on the non-toxic parts of toads [105]. Socially acquired prey preferences can also be modified later in life [106]. 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 [107]. This type of reversal learning, wherein predators adjust their prey choices based on stimuli, is important when thinking about the identification and fitness reward of auto-mimics (e.g. monarch butterflies that lack cardenolides). If predators could enhance their identification of prey profitability through social transmission, then they could influence how frequency-dependent selection operates on prey [104]. Because social transmission of avoidance is beneficial for defended prey [104] we would expect selection to favour prey to evolve traits that maximize opportunities for social learning about identification such as new, perhaps more salient, multi-modal defences [108] that increase distastefulness to elicit strong disgust responses [109]. 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.

5.3. 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 [110–112], mammals and birds [105,113,114], and even in limbless predators such as snakes [115]. At first glance, dissecting behaviour

is a surprising evolutionary solution for snakes. However, it is made possible because of the enlarged posterior maxillary teeth [115] 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 [116]. Dissecting behaviour is innate in some mustelids [78,117,118], 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 [17,92]. Dissecting behaviour may evolve and be maintained via cultural transfer [105] because headshaking in response to aversive stimuli could be used by conspecifics to guide dissecting behaviour [119], and for individuals to develop discriminatory chemosensory behaviour [17].

The widespread occurrence of dissecting behaviour suggests a shared ability to taste and avoid CTS in predators [120]. Although cardenolides are often described as bitter tasting compounds [121], we lack specifically designed comparative tests on the chemosensory detectability of CTS. Japanese tiger keelback snakes (*Rhabdophis tigrinus*) show no discrimination between purified bufadienolides and control stimuli [122], 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 [17]. 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 [34]. 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 coevolution.

Some predators, such as *P. 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 [30]. This taste insensitivity may be an adaptation to let predators consume CTS-defended prey, and there appears to be sufficient intraspecific variability in this behaviour to have resulted from natural selection, but this is yet to be investigated [28]. Taste insensitivity to cardenolides suggests that either the taste receptor genes have undergone functional changes, or that the valence of CTS has changed, or can be changed, from negative to positive. Future research comparing the comparative responses of predators combined with comparisons of the g-protein-coupled Tas2r taste receptors responsible for bitter taste perception could reveal patterns of evolution related to prey defences and predator diet, and test whether taste insensitivity is paired with a detoxifying metabolism or target-site insensitivity (TSI) [123,124].

5.4. Consumption

Evolved avoidance of CTS by dissecting or eating the least CTS-laden parts of prey is one possible result of predator–prey interactions. In this section, we describe TSI via amino acid substitutions in the CTS binding pocket of the NKA and its potential as a candidate for predator–prey coevolution. If an arms race-type process is occurring, we expect matched levels of CTS defence of prey and resistance ability of the predator [9].

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 first extracellular loop (residues 111–122; H1–H2 loop), which makes up part of the CTS binding domain and shows clade- and paralog-specific patterns of variability but also shows remarkable patterns of convergence, parallelism and divergence [125]. 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 [21] and have evolved in snakes [63,126], frogs [127,128] and other vertebrates [125].

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 [129]. Snakes that have shifted their diet from eating toads to eating fireflies do have TSI [54]. It has been hypothesized that the black-headed grosbeak which feeds on monarch butterflies also possesses amino acid substitutions in two of the three paralogs which likely confer resistance [31,130]. In other species of birds that are reported as specialist feeders of CTS-defended danaid butterflies [131], 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 [31].

Understanding the evolutionary history and potential for coevolution of a trait requires some knowledge of the patterns of variation among individuals, populations and species [132]. 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 [125], 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 [31,133]. 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.

6. 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 [23,37,38] from species to species and within populations [44–49], in terms of concentration, diversity [10] and polarity, which can influence their chemosensory detectability [17], toxicity [134], transport [135] and excretion [136]. CTS also vary seasonally and geographically [137,138], which may influence selection for TSI. In this section we draw on the information from plant–herbivore interactions where insects that possess sensitive NKA still feed on cardenolide-defended plants [36,139,140]. We discuss how predators could possess guts that are impermeable to cardenolides via biological barriers [141,142]; how hormonal systems can mitigate loss of NKA activity; and the scope for gut microbiota to neutralize the toxicity of CTS (see figure 3).

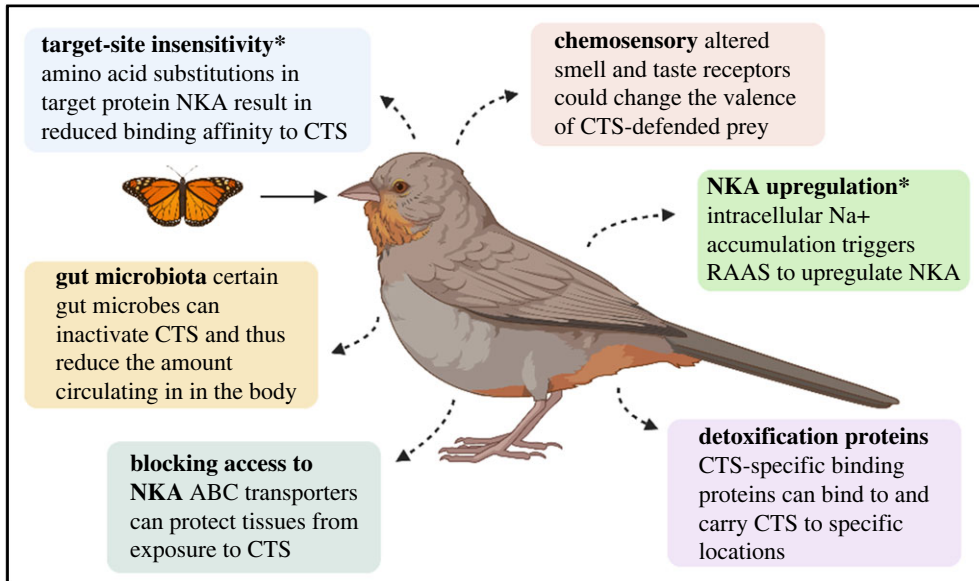## 6.1. ATP-binding cassette transporters and binding proteins

One method to avoid CTS toxicity which has not been explored in predators is having an impermeable barrier to non-polar CTS [144,145]. 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 [135]. 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 with wild-type mice [146].

Binding proteins could also contribute to CTS resistance in predators [147]. 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 [11,148]. Because endogenous CTS function in regulating cardiac contractility and circulation [20], 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 [149,150]. These binding proteins are produced at high concentrations in the kidneys, where they probably protect the NKA of those tissues [148]. 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.

6.2. Renin–angiotensin–aldosterone system 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 [151], 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 [152]. 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) [155,156]. Increased circulating aldosterone has been identified in the Japanese toad-eating snake *R. tigrinus* [152], which exhibits highly enlarged adrenal glands [151]. Furthermore, garter snakes (*Thamnophis elegans*) injected with bufadienolides responded

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 (e.g. [120]) or detoxify CTS after ingestion (e.g. [143]). In addition, they may possess altered target sites that are no longer susceptible to the toxic action of CTS [125]. Some predators sequester CTS from their prey and defend themselves against their own predators (e.g. snakes of the genus *Rhabdophis* [15]). 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.
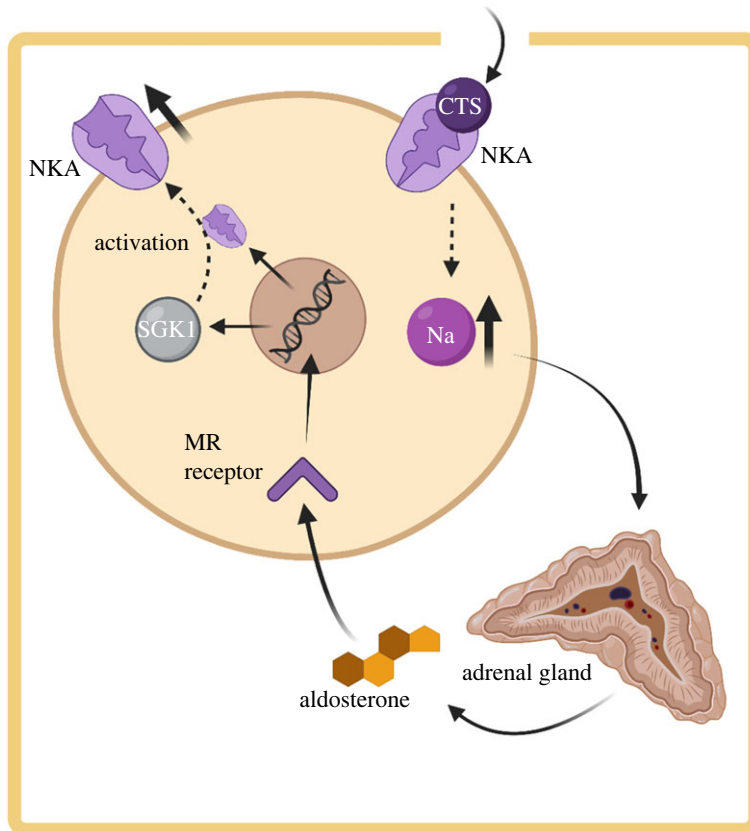
with significantly increased NKA expression in their heart tissue [157]. However, whether CTS exposure directly leads to increased circulating aldosterone and NKA expression, and consequently adrenal gland enlargement in resistant predators, requires further experimental tests.

6.3. Gut microbiota

Gut microbiota are known to neutralize the toxicity of CTS by metabolizing CTS to reduced/inactivate compounds such as digoxin to dihydrodigoxin [143]. 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) [143,158]. 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 includes eight genes, which are present in individuals that can metabolize 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*) show that it is sufficient for digoxin inactivation [159] 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, remain open questions.

7. Benefits and costs of consuming cardiotonic steroids

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 §§5 and 6 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 [160] as seems to be the case with the

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 (MR) hormone aldosterone, which passes through the cell membrane and binds to an intracellular 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 [153,154].

population of scansorial black-eared mice (*P. melanotis*) that are larger, heavier and reproduce more than mice of the same species whose territories are outside of the overwintering monarch roosts [27]. Thus, in this section, we cover the specific aspects of predator counter-adaptations to CTS, propose a range of putative benefits and costs, and suggest how these could be measured.

7.1. 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 [161]; African crested rats (*Lophiomys imhausi*) have hairs that are highly specialized to wick up and store the cardenolide that they chew from the roots and bark of *Acokanthera schimperi* (Apocynaceae) [42]. 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. Similarly, Japanese tiger keelback snakes (*Rhabdophis tigrinus*), which store bufadenolides in specialized nuchal glands on the back of their necks [15], arch their necks towards the threat revealing brightly coloured yellow and red skin covering the nuchal glands [162]. 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 S.M.). Japanese tiger keelback snakes also maternally provision bufadenolides to their offspring via embryonic transfer. Gravid female snakes have been found to actively forage for toads [163], and the amount of CTS in the nuchal glands of offspring corresponds proportionally to the amount found in the mother [164]. 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 [54]. 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 [165], is an open question. This is possible, given that other species of grosbeak appear to have toxins in their feathers [166], 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 [64,167–169]. The behaviour is not exclusive to bufophagous snakes, and at least one species of highly bufophagous snake (*Causus rhombeatus*) does not feign death [170]. However, the enlargement of the adrenal glands in several species of bufophagous snakes [151] is thought to be linked to this behaviour. Increased catecholamine production by enlarged adrenal glands could lead to a parasympathetic syndrome preceding death feigning [170]. Because CTS may render toad-eating snakes distasteful, 'death feigning' may slow a predator's attack and increase the predator's detection of CTS [171].

Beyond chemical defence sequestration, predators may take advantage of CTS to protect themselves from parasites [172,173]. 'Self-medication' [174] has not been investigated in predators that feed on CTS, but the diverse pharmacological properties of these compounds suggest that such an evolutionary relationship is possible. For example, several bufadienolides have been shown to have antimicrobial and antifungal properties [49,175]. Further, monarchs are well known to use cardenolides for self-medication, whereby they protect their eggs from parasites by laying them on CTS-defended milkweed plants [176].

7.2. 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 [177]. Otters, for example, can ingest frogs immediately but require more time to skin, wash and select the parts to ingest from a toad [113]. Black-headed grosbeaks (*Pheuticus melanocephalus*) and orioles (*Icterus parisorum*) that feed on monarch roosts feed on a 7.85 day on-off cycle [102], and also change their feeding depending on ambient temperature [102], which is probably due to the changes in toxicity with ambient temperature [178,179]. 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 wild-type *ATP1A1* onto *ATP1A2* negatively affects their learning ability, locomotor activity and anxiety-related behaviours [180]. 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 with non-toad eaters [181,182].

Endowing a protein with a new function through mutation often incurs a cost, particularly with respect to the protein's original function [125,128,140,183]. Functional studies of TSI have repeatedly shown that resistance-conferring substitutions often carry substantial functional costs to the ATPase activity of NKA [125,128]. These negative pleiotropic effects can have major implications at higher biological levels due to the vital role that NKAs 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 [128,140,183] or, as is the case with neotropical grass frogs of the genus *Leptodactylus* (Leptodactylidae) that feed on toads (but do not specialize on them), undergone a tandem duplication of *ATP1A1* and subsequent neofunctionalization of one copy, which allows them to maintain highly resistant and highly functional versions of the protein [128,133].

8. A broader view of cardiotonic steroids resistance in predators and the coevolution with cardiotonic steroids 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 have occurred in mammals and birds; and that TSI via amino acid substitutions in the CTS binding pocket of the NKA has evolved in parallel in invertebrate and vertebrate predators. We have also

pointed to biochemical, hormonal and microbiological strategies that have yet to be investigated in this context.

Using the predation sequence as a framework [1], 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 [184–186]). This difference in the selective pressure between the early and late stages of predation supports a pattern predicted by Endler [1], which is a tendency for many generalized predation methods to have evolved in the early stages of predation (optimal foraging, social learning, dissecting behaviour, changes in gustatory perception). There are three potential reasons for this. (i) Generalized methods may be less expensive than specialized 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 [187]. 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 [188]. In many cases, predators choose prey on the basis of their overall availability and profitability [189,190]. (ii) Because prey defences that operate early in the sequence are generalized and only generalized 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. [9]). Finally, (iii) the interactions between predators and CTS-defended prey are diffuse due to the community complexity of these natural predator–prey systems [191]. Our analysis shows that most predators prey on several species, and therefore the total selective pressure on each other is more diffuse.

Understanding the evolutionary history and potential for coevolution 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 specialization on—or tolerance to—CTS-defended prey will be important for understanding whether these animals are pre-adapted to attack CTS-defended prey [29,192] or whether TSI evolved directly from exposure to CTS, and whether there is evidence for ongoing coevolution.

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

question	experimental scheme(s) to address question
chemosensory	
How do the taste receptor genes of CTS-resistant predators compare with those of sensitive predators?	Comparing the Tas2r genes of *Peromyscus* species that have varying sensitivity to cardenolides compared with related species of mice would reveal the underlying molecular mechanisms of CTS tolerance.
Are predators that dissect able to chemically identify CTS-laden tissue?	Modifying either real or artificial CTS-defended prey so that the CTS 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.

Table 1. (*Continued.*)

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question	experimental scheme(s) to address question
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 [193]. The genes encoding these proteins fall into seven subfamilies (A–G) and have ancient eukaryotic origins [194]. ABCG2 or ABCG2-like genes have been found in 41 bird species, and ABCG2-like genes have been lost in only five species [195]. 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 [196] and such studies would confirm their ability to protect tissues. Exploring the coevolution 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 CTS?	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 versus non-resistant predators would reveal whether they play an adaptive role in predators of CTS-defended prey.
physiological mechanisms of resistance	
Does the RAAS play a role in CTS resistance?	Rearing hatchling CTS-resistant animals (snakes or mice) on a diet with and without CTS 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 TSI.
Are there physiological costs to feeding on CTS?	Comparing the physiology and performance of CTS-resistant predators fed CTS-defended prey (toads) versus control prey (non-toad frogs) would reveal whether digesting the compounds is physiologically demanding and provide insights into the cost of this adaptation.

(*Continued.*)

Table 1. (*Continued.*)

question	experimental scheme(s) to address question
role of gut microbiota	
How widespread are gut bacteria that can digest CTS 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 non-resistant 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 non-resistant 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 [197,198]. 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.

9. Conclusion 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 TSI of NKAs, we have found that there are multiple physiological, chemosensory, behavioural and ecological mechanisms that can also contribute to, and consequently shape, the ability to overcome the CTS defence of prey. 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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