Myth busting – cane toads in Australia

The cane toad is an invasive pest that is colonising northern Australia and poisoning native predator species. Studies into cane toad chemical ecology have busted many myths and revealed the promise of new control solutions.

The cane toad (*Bufo marinus*) was introduced into the sugar-cane growing regions of northern Queensland in 1935, in an unsuccessful attempt at biocontrol of the sugar-cane beetle (*Dermolepida albohirtum*).[1] The planning and forethought behind this endeavour seem, at least by modern standards, woefully inadequate. Not only did cane toads fail to control the sugar-cane beetle, which was eventually achieved some years later by the insecticide gammaxene,[2] but the toad itself went on to become an invasive pest of epic proportion – far outliving and exceeding the sugar-cane beetle threat. Since the toad's release, the invasion front has advanced south along the eastern seaboard from Queensland into northern New South Wales, and west through the Northern Territory to a likely 2009 crossing into Western Australia – leading to the colonisation of more than one million square kilometres.

Cane toads are renowned for their ability to produce and deploy cardiotoxic steroids, known as bufadienolides, as a form of chemical defence. Bufadienolides are antagonists of Na^+/K^+ ATPase in much the same way as the plant-derived cardenolides, such as digitalis. Many Australian native predator species, including freshwater crocodiles, marsupials, snakes and lizards, are vulnerable to cane toad poisoning, with ingestion of bufadienolides leading to cardiac arrest and death. The prospect of such fatal encounters with cane toads is further enhanced by the presence of specialised parotoid glands that secrete high concentrations of bufadienolides in response to predatory attack. If this was not enough, these same glands exude high concentrations of adrenaline – providing a double blow to the cardiovascular system of poisoned animals. As the cane toad invasion front advances across Australia, native predator populations are continually challenged.

Current control strategies

Historically, cane toad control strategies can be viewed as either *physical* or *biological* approaches. The *physical* approach favours a low-technology, localised containment solution designed to inhibit or delay the movement/presence of toads by using barriers and traps, and through the use of hand-collection campaigns. While immediately deployable, and locally effective, physical strategies are labour intensive, target only a fraction of the colonisation area, and at best only delay the inevitable. They can, however, inspire and engage local communities against a common threat, and attract media attention that serves to alert the public to the cane toad problem. Physical strategies are best characterised as a delaying tactic, buying time for the development of more effective longer-term solutions. The *biological* approach seeks to develop and deploy lethal self-replicating biological vectors, such as viruses or parasites, with or without genetic modification. Although capturing the public imagination for 'the perfect' scientific fix, the reality of biological control has (so far) been more promising than substance. As any biological solution by definition lacks a reliable 'product recall' option (consider the release of cane toads themselves), it is critical that it be species specific, effective and without serious adverse side effects – a guarantee that has so far proved impossible to satisfy. The development of biological solutions suffers from a very high risk of scientific and technical failure, and a high cost and extended time to develop and deploy. This latter point is all the more concerning given the pace of the toad invasion front, which could complete its crossing of northern Australia, from the Pacific to Indian oceans, by 2020. A biological solution that takes an additional 10 years to develop (if at all) would be a Pyrrhic victory indeed! A further challenge to the biological solution is the need to quarantine the effect to Australian cane toads, so as to not adversely impact toads in other parts of the world, including adjacent Indonesia.

Cane toad control in Australia has been dominated by limited, short-term and localised success with physical solutions, and the unrealised promise of biological solutions. Clearly, the cane toad is a formidable adversary, capable of using chemistry to enhance survival and wreak havoc among Australian predator species. Faced with the inevitable colonisation of northern Australia, and in the absence of effective control strategies, we proposed a new approach to cane toad control – the search for a *chemical* solution.[3] In 2006, and with contract research support from the Queensland Government via the Invasive Animals CRC, we undertook a two-year study of the chemical ecology of the cane toad in Australia, with a view to identifying promising new directions for control.[4] While the initial focus was on pheromones and

toxins, towards the end of this study we expanded the brief to examine cane toad bacteria – leading to what we believe was a significant discovery that holds out the promise of a combined *chemical/biological* solution.

Myth busting

As with any new research project, we reviewed all available literature to establish what was actually known, and used this to identify gaps in our knowledge of cane toad chemistry in the Australian setting. To our surprise, we found a landscape devoid of detailed analysis of Australian cane toad chemistry, but one rich in pseudo-knowledge coloured by an almost irrational fear and loathing of toads, further compounded by the use of emotive language, a ready acceptance of myths and half-truths, and a barbaric enthusiasm to apply control solutions (cricket bats, golf clubs…) that would make any animal ethics committee wince. Cane toads really do have a PR problem! To set the record straight…

- *Cane toads spit venom*: False. Venoms are more typically associated with a delivery apparatus, such as fangs or spines, all absent in toads. Likewise, toads lack the anatomical features needed to spit (or fire) toxin. Cane toad toxins are exuded from skin glands (predominantly the parotoid glands) in response to predatory attack, and as such are better characterised as defensive secretions, not venoms.
- *Cane toads poison waterways*: False. Toads are poisonous, but there is no evidence that they poison waterways any more than any of the large number of poisonous Australian native species. It is also worthwhile noting that cane toad bufadienolides are not water soluble.
- *Cane toads spread disease*: False. Again there is no evidence that cane toads are vectors for human or animal pathogens, any more or less than other animals.
- *Cane toad toxins are cardioactive glycosides*: False. This misinformation appears to stem from non-chemists using chemical terminology without understanding its true meaning. While the toad toxins are rich in cardiotoxic steroidal bufadienolides, none of these is a glycoside.

The cane toad 'toxin'

In our review of the cane toad literature (scientific and lay), we often encountered reference to the 'cane toad toxin' as a single chemical entity, demonstrating a failure to see the toxin as a mixture of chemicals, each with differing biological properties, potency and selectivity – collectively contributing to an ecological role. This simplistic categorisation of the 'toxin' sidestepped a chemically informed analysis and appreciation of its true ecological role – a serious oversight given that the 'toxin' is *the* underlying cause of the environmental problem. Our chemical analysis of Australian cane toad toxins revealed a complex mixture of 50-100 bufadienolides in adults, with a comparable diversity in eggs and early stage tadpoles and almost zero overlap in molecular diversity between adults and eggs/tadpoles (Fig. 1).[5] Of note, late-stage tadpoles lack any bufadienolides and, contrary to popular belief, are apparently non-toxic to vertebrates (but very toxic to insects[6]), which begs the question of how we actually define toxicity. Again, popular commentary on cane toad toxicity is often simplistic, presenting measures without due regard to how the toxin was collected and processed, or what species were used to test a toxic response, and how. For example, toxicity testing cane toad eggs against Australian frog tadpoles would be likely to return different results from testing adult cane toad parotoid secretions against mice, dogs or Na^+/K^+ ATPase preparations from pig kidneys (the standard source of this enzyme). Furthermore, different bufadienolides within toxin preparations will exhibit differing binding constants on differing Na^+/K^+ ATPases, depending on tissue (kidney, heart…) and species (pig, quoll, lizard, crocodile…), so that measures of toxicity need to be qualified by which bufadienolide(s) against which Na^+/K^+ ATPase(s).

Alarm pheromone

But what of chemical control solutions? Despite considerable efforts, to date there is no compelling evidence for a behavioural response consistent with a cane toad sex pheromone. That said, the case for a cane toad tadpole alarm pheromone is strong. Damaged tadpoles release a chemical cue into the water that triggers an immediate flight response in adjacent conspecifics (Fig. 2). This cue is species specific, non-toxic, biodegradable (or at least easily diluted) and extremely potent. More importantly, cane toad tadpoles chronically exposed to crushed tadpole extract undergo premature metamorphosis to form underweight metamorphs, with presumably a lower probability of survival and lifetime reproductive success.[7] Our preliminary studies suggest that the alarm pheromone is a low molecular weight water-soluble organic molecule. This raises the possibility that addition of synthetic tadpole alarm pheromone to controlled waterways (dams, lakes, streams, irrigation canals) during the breeding season could disrupt normal cane toad development and reduce generational recruitment. While not a national eradication solution, a control solution based on the alarm pheromone could be a valuable management tool.

Microbial biotransformation

Encouraged by the prospect of an alarm pheromone solution, we turned our attention to cane toad toxins. As noted above, we established that the bufadienolide content in Australian cane toad parotoid glands is far more diverse (50-100 bufadienolides) than previously assumed, at least compared to overseas reports (4 bufadienolides).[8] We speculated that a broadening of the parotoid gland bufadienolide chemical

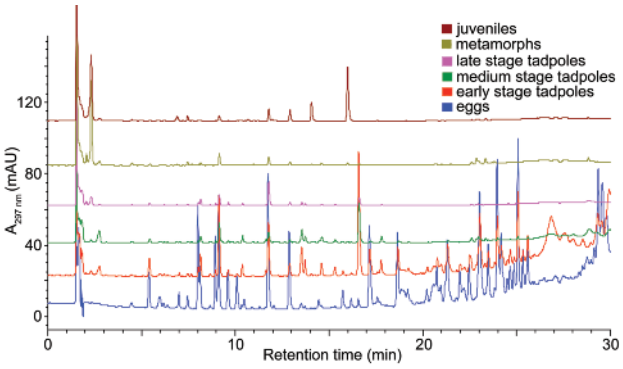

Figure 1. HPLC traces (297 nm) of *n*-BuOH extracts from studied ontogenetic stages of cane toads analysed at 297 nm. The α-pyrone ring of bufadienolides has a distinctive absorption maximum at 297 nm.

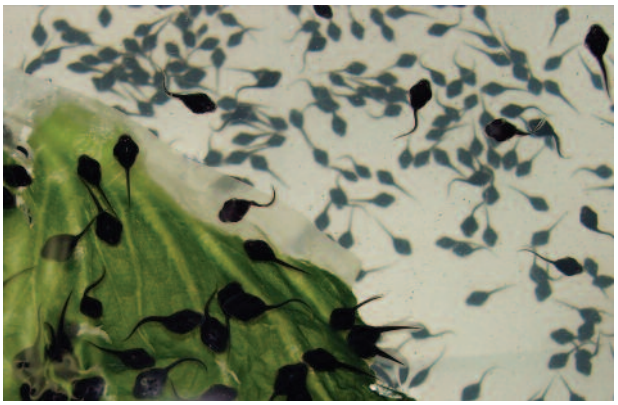

Figure 2. Cane toad tadpoles flee rapidly from a water extract of a crushed conspecific. The response is thought to have evolved, since the only time the bodily fluids of a tadpole will be in the water is after they have been attacked by a predator.

diversity could lead to survival advantages by increasing the prospects for antagonism of a wider array of Na^+/K^+-ATPase isoforms. Binding constants for bufadienolides against Na^+/K^+-ATPase can range over several orders of magnitude, demonstrating that even minor bufadienolides could have ecological significance against relevant species.[9] Accepting the premise that cane toads may benefit from more chemically diverse toxins raises the challenge of how this diversification could have happened. It is known that bufadienolides can undergo biotransformation when exposed to laboratory cultures of bacteria or plant cells. We speculated that cane toads may have co-opted bacteria to diversify and calibrate the bufadienolide content of their toxin, to achieve a defensive outcome optimised against Australian native predator species. To test this hypothesis, we cultured bacteria from dissected cane toads, and confirmed that a parotoid gland strain of the gram-negative bacterium *Comamonas testosteroni* was indeed capable of biotrans-

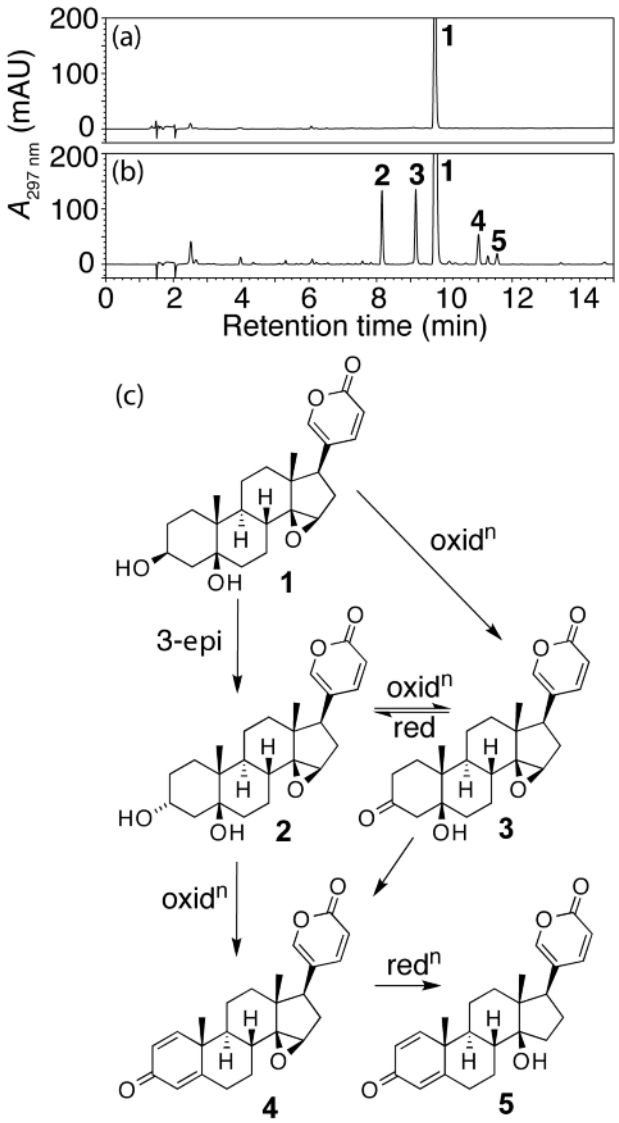

Figure 3. Bacterial biotransformation of the bufadienolide marinobufagin (**1**) by a strain of *Comamonas testosteroni* isolated from cane toad parotoid gland secretion. HPLC traces (297 nm) of ethyl acetate extracts obtained from (a) uninoculated nutrient broth containing **1** and (b) nutrient broth inoculated with *C. testosteroni* containing **1**, both after incubation at 26.5°C for 96 hours. (c) Biotransformation products 3-*epi*-marinobufagin (**2**), 3-oxomarinobufagin (**3**), $\Delta^{1,4}$-3-oxoresibufogenin (**4**) and $\Delta^{1,4}$-3-oxobufalin (**5**), and a possible sequence of transformations.

forming bufadienolides (Fig. 3).[8] This represented the first account of biotransforming bacteria within an anuran.

The prospect that bacteria may play a role in the chemical ecology of the cane toad – modifying toxicity and environmental impact – suggests an exciting new line of research for cane toad control. More detailed knowledge of the relationship between cane toads, bacteria and bufadienolides could, we believe, contribute to our ability to control the impact of cane toads on native predator species. For instance, more competitive strains of *C. testosteroni* (or other microorganisms) that either have no capacity for biotransformation or over-transform all available bufadienolides to non-toxic analogues, could be used to supplant wild strains – a probiotic approach. Alternatively, cane toads could be infected with bacteriophages specific to bufadienolide-biotransforming bacteria, thereby down-regulating toxicity, and lessening environmental impact. Even if one or more of these approaches does not reduce cane toad numbers directly, should they succeed in lessening toxicity to the point where it is non-lethal to Australian native predators, then either the predators will consume cane toads with impunity (an ironic and satisfying turn of events) or, at the very least, predators will recover from non-lethal poisonings and acquire the learned behavioural response of leaving the toads alone.

Future directions

While it is still too early to judge whether our investigations into cane toad chemical ecology will inspire the development of practical control solutions, there is cause for hope – a step forward in what has otherwise been seen as a hopeless situation. Furthermore, should such a chemistry-inspired solution emerge, it will be a testament to the need to support a strong and vibrant Australian chemistry research capability, and the value of engaging in multidisciplinary collaboration.

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