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A Critique of the Toxicoferan Hypothesis

Authors:

Adam D Hargreaves[1], Abigail S Tucker[2] and John F Mulley[3]

1. Department of Zoology, University of Oxford, South Parks Road, Oxford, OX1 3PS, United Kingdom

2. Department of Craniofacial Development and Stem Cell Biology, King's College London, Guy's Hospital, London SE1 9RT, United Kingdom

3. School of Biological Sciences, Bangor University, Deiniol Road, Bangor, Wales, LL57 2UW, United Kingdom

Author email addresses:

adam.hargreaves@zoo.ox.ac.uk

abigail.tucker@kcl.ac.uk

j.mulley@bangor.ac.uk

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Abstract

Historically, venom was believed to have evolved twice independently in squamate reptiles, once in the advanced snakes and once in venomous lizards. The presence of putative toxin proteins in the saliva of species usually regarded as non-venomous, and the expression of venom gene homologs in their salivary glands, led to the hypothesis that venom evolved a single time in reptiles. As the single, early origin of venom is synonymous with the Toxicofera clade (Serpentes, Anguimorpha and Iguania), it will subsequently be referred to as the Toxicofera hypothesis. This hypothesis has proved to be remarkably pervasive for almost a decade, but has until recently never been tested. Here, evidence used in support of the Toxicofera hypothesis is reviewed and critically evaluated. Taking into account both new and old data, it appears that this hypothesis is unsupported, and should be subject to further scrutiny and discussion. Finally, the implications of the rejection of the Toxicofera hypothesis are discussed, with respect to the knowledge of venom evolution in the Reptilia and also the practical implications of this knowledge.

Introduction

Venomous reptiles have long been the source of fear and fascination in roughly equal measure, not least because of the extensive annual global mortality and morbidity caused by reptile envenomation, particularly in the developing world (Kasturiratne et al. 2008; Harrison et al. 2009). Research effort has traditionally focused on the characterisation of venom toxins and the development of treatments to counteract their clinical effects, and so species considered to be medically important have received the most attention (for example, the saw scaled vipers (Wagstaff and Harrison 2006; Wagstaff et al. 2009; Casewell et al. 2009)). As a consequence, the full evolutionary history of venom in the Reptilia has remained unknown, and to this day poses unanswered questions, including fundamental topics such as the origin of venom toxins,

what constitutes venom and a venomous animal and even the timing of the evolution of venom itself.

Hypotheses concerning the evolution of venom within reptiles have undergone dramatic revision within the last decade, and are currently in a state of flux. Historically, venom within reptiles was believed to have evolved twice independently: once in the Caenophidia (advanced snakes) and once in the Helodermatid lizards (Gila monsters and beaded lizards) (Kochva 1978; Pough et al. 2004) (Figure 1). This belief was mainly due to the distant phylogenetic relatedness of these animals and clear differences in the morphology of their respective venom delivery systems (Kochva 1978; Saint Girons 1988). A more recent, alternative hypothesis (which we refer to as the "Toxicofera hypothesis") has become widely accepted within (and seemingly far beyond) the toxinological community. The Toxicofera is a clade of squamate reptiles comprising Iguania, Anguimorpha and Serpentes, whose name refers to the presence of venom within at least some members of these groups (Vidal and Hedges 2005). Phylogenetic analysis utilising nine nuclear genes (*α-enolase*, *amelogenin*, *c-mos*, *hoxa13*, *jun*, *mafb*, *rag1*, *rag2* and *r35*) found this clade to be strongly supported (Vidal and Hedges 2005), and this support has been reproduced in subsequent studies (e.g. Pyron et al. 2013). However, phylogenetic relationships within the Toxicofera are unresolved based on nuclear data, although the use of SINEs (short interspersed nuclear elements) has suggested a clustering of snakes with anguimorph lizards (Piskurek et al. 2006) which is also supported by a more recent analysis (Hsiang et al. 2015).

FIGURE 1

Figure 1. Simplified Reptile cladogram. The phylogenetic position of venomous Helodermatid lizards and the Caenophidia (advanced snakes) are indicated. The phylogenetic position of the

proposed venomous Toxicoferan ancestor is indicated along with the three proposed punctuated toxin gene recruitment events. Proposed recruited toxin gene families are also shown.

The majority of the roughly 2,500 species of snake are classified within the Caenophidia, a sub-order containing four major lineages: Atractaspidinae; Viperidae (vipers, pit vipers); Elapidae (such as cobras and mambas) and Colubridae (a polyphyletic group which is constantly undergoing taxonomic revision) (Quijada-Mascarenas and Wüster 2009). Approximately 600 species, all belonging to the former three lineages, were traditionally considered to be venomous in that they possessed venom glands surrounded by compressor muscles, tubular fangs at the front of the mouth and are of medical significance to humans (although medical significance to humans is obviously a poor criterion on which to base classification of toxicity). Whilst some members of the Colubridae are opisthoglyphous (rear fanged), they do not generally pose a threat to humans and have historically not been considered to be venomous.

Evidence for a wider use of venom within advanced snakes was initially based on proteomic analysis of the saliva of the radiated rat snake (*Coelognathus radiatus*), a snake reliant on constriction for prey capture, where a post-synaptic neurotoxin belonging to the three finger toxin (3Ftx) family was discovered (Fry et al. 2003a). This protein was found to possess the typical ten conserved cysteine residues of elapid 3Ftxs and when functionally tested led to antagonism of nicotinic acetylcholine receptors. This protein was therefore considered to be structurally and functionally homologous to the elapid three finger toxins (Fry et al. 2003a) and phylogenetic analysis showed strong support for the nesting of the rat snake 3Ftx within a clade of previously categorised 3Ftxs (Fry et al. 2003b). On the basis of these results it was suggested

that three finger toxins were recruited into the venom repertoire prior to the divergence of the Elapidae and Colubridae (Fry et al. 2003a). Indeed, the analysis of other colubrid "venoms" (Mackessy 2002) added further support that the use of venom in the advanced snakes pre-dated their radiation in the Cenozoic era (Vidal and Hedges 2002). More interestingly, the presence of putative toxin proteins in the saliva of lizard species usually regarded as non-venomous (such as the lace monitor, *Varanus varius*), and the expression of venom gene homologs in their salivary glands, led to the proposed hypothesis that venom evolved a single time in squamate reptiles approximately 170 Mya (Fry et al. 2006), and not twice independently as had been previously believed (Pough et al. 2004; Kardong et al. 2009).

The timing of venom gene recruitment events within reptiles has undergone significant modification over the course of subsequent Toxicofera-related studies, with further sampling leading to the detection of an increased number of putative venom genes in a diverse collection of species (Fry et al. 2009; Fry et al. 2010; Fry et al. 2012a; Fry et al. 2013). These findings suggest an increasingly complex view of venom gene recruitment throughout the evolution of the Toxicofera, which has even extended to include the Komodo dragon (*Varanus komodoensis*). This species was previously considered to be reliant on oral bacteria (e.g. see Bull et al. 2010) to induce septicaemia in prey items, but is now considered to be venomous (Fry et al. 2009).

Here, the foundation and expansion of the Toxicofera hypothesis and the proposed single, early evolution of venom in reptiles are discussed and examined. The assumptions and key shortcomings of the evidence used in support of this hypothesis are reviewed, taking into account more recent findings and novel interpretations.

The Toxicofera hypothesis

The first proposal of the single, early origin of venom in reptiles occurred in 2006 based upon the detection of genes homologous to those previously identified in the venom glands of

venomous snakes expressed in the mandibular salivary glands of four Varanid lizards (*Varanus acanthurus*, *V. mitchelli*, *V. panoptes rubidus* and *V. varius*) and a single Iguanian (*Pogona barbata*) (Fry et al. 2006). Phylogenetic analysis demonstrated that nine toxin families were shared between these non-venomous lizards and advanced snakes: AVIT peptide; B natriuretic peptide; cysteine-rich secretory protein (CRISP); cobra venom factor (which is in fact complement component C3 (Alper and Balavitch 1976)); crotamine; cystatin; kallikrein; nerve growth factor and vespryn. Additionally, a type III phospholipase A_2 (PLA_2) was detected in the mandibular salivary glands of *Varanus varius* (Fry et al. 2006).

Subsequent Toxicofera-related studies mainly focused on the inclusion of additional lizard species (Fry et al. 2009; Fry et al. 2010; Fry et al. 2013). A more recent study sequenced cDNA derived from the oral glands of Iguanian lizards and Henophidian snakes using 454 pyrosequencing (Fry et al. 2013). The detection of apparent homologs of several Toxicoferan genes in these species led to a number of proposed gene recruitment timing events being shifted even earlier in Toxicoferan evolution, in some cases by up to 112 million years, and the adoption of a punctuated evolutionary history of toxin recruitment. In this scenario, three rounds of toxin gene recruitment have been proposed to have occurred in the Toxicofera: up to ten at the base of the Toxicofera (cysteine-rich secretory protein (CRISP), crotamine, cystatin, cobra venom factor, kunitz, L-amino acid oxidase, lectin, renin aspartic protease, veficolin, vespryn), six in the ancestor of Serpentes and Anguimorpha (AVIT peptide, epididymal secretory protein, hyaluronidase, kallikrein, nerve growth factor, ribonuclease) and eight (acetylcholinesterase, lipocalin, C-type natriuretic peptide, snake venom metalloproteinase, phosphodiesterase, phospholipase B, vascular endothelial growth factor, waprin) in the common ancestor of the Caenophidia (Fry et al. 2013) (Figure 1).

The Toxicofera hypothesis proposes the existence of an early venomous squamate that would have possessed toxin-secreting glands on both the upper (maxillary) and lower (mandibular)

jaw (Fry et al. 2006). The venom delivery systems in advanced snakes and lizards are therefore homologous but morphologically distinct derivatives of this primitive system, with snakes retaining the maxillary venom glands and venomous lizards maintaining the mandibular glands (Fry et al. 2006), with the opposing glands being secondarily lost by each lineage. It has been proposed that members of the Iguania (such as the green anole lizard, *Anolis carolinensis*) diverged whilst this venom system was in an incipient stage, and so lack any form of specialised toxin secreting glands. Furthermore, snakes which use alternative prey capture methods such as constriction are proposed to have secondarily lost venomous function (Fry et al. 2006).

Alongside the conserved shared expression of homologous genes, the conserved structure of homologous proteins has also been used to support the Toxicofera hypothesis, namely the conserved cysteine structure and functional residues (Fry et al. 2006).

Several Toxicofera-related studies have also included functional tests on the mandibular oral secretions of two varanid species, *Varanus komodoensis* and *V. varius* (Fry et al. 2006; Fry et al. 2009). Samples of crude oral secretion and purified natriuretic peptide were injected intravenously into anaesthetised male rats, which resulted in a drop in mean arterial pressure (MAP). Platelet aggregometry was also carried out using purified type III PLA_2 from *V. varius* which showed inhibition of platelet aggregation when tested on human blood samples.

Shortcomings of the Toxicofera hypothesis

The Toxicofera hypothesis assumes that shared expression of a gene between what were previously considered non-venomous species and more derived venomous species implies shared toxicity (or at least a shared venomous ancestry) (Fry et al. 2006). It is of course plausible that homologous tissues (e.g. the venom gland and other oral glands) within related species will express similar complements of genes, and therefore presence alone does not provide any evidence of toxicity. Indeed, many of the proposed toxins which have been used

to support the Toxicofera have never been functionally characterised. Moreover, the products of several of these genes have never been suggested to be toxic (for example cystatin type E/M (Ritonja et al. 1987)) or have been shown to not be toxic, even up to high doses, through functional tests (for example, acetylcholinesterase (Cousin et al. 1996)). Therefore these genes have been used to support shared ancestral toxicity, without actually functioning as toxins. Additionally, it now seems certain that many of the proposed shared venom toxins within the Toxicofera actually results from the confusion of orthologs and paralogs, where non-toxic relatives of toxin genes have been identified (Hargreaves et al. 2014a). For example, genes encoding complement c3 and nerve growth factor have been shown to have undergone an Elapid-specific gene duplication (Sunagar et al. 2013; Hargreaves et al. 2014a; Hargreaves et al. 2014b) to give rise to the putatively toxic "cobra venom factor" and nerve growth factor b (Hargreaves et al. 2014b). This mis-identification of physiological orthologs as toxin-encoding paralogs has led to the conclusion that all Toxicoferan reptiles produce toxins in their oral secretions, and are therefore descended from a common venomous ancestor. In addition, many previous studies (e.g. Casewell et al. 2012) have been based on a flawed assumption – that phylogenetic trees containing monophyletic clades of *reptile sequences* that include a known (or hypothesised) toxin from venomous snakes constitute *venom toxin clades*. The true evolutionary history of these genes (which have duplicated to possibly give rise to toxic versions in *some* species), and these clades (which contain both genes encoding toxic products in *some* species, along with related genes encoding non-toxic products in other species), has therefore been obscured by being labelled as toxins by default. This is further confounded by a lack of data, both for the tissue being studied and also for other tissues and species (the majority of Toxicofera-related studies (Fry et al. 2006; Fry et al. 2010; Fry et al. 2012a) used only "up to 384" individual venom gland cDNA library colonies per species, a minimal amount of sequencing considering the frequently cited complexity of snake venoms (Li et al. 2005b;

Kini and Doley 2010; Casewell et al. 2013)). This paucity of data, whilst understandable given the technology and resources of the time, has seemingly led to errors of interpretation, and, possibly more seriously, over-interpretation of results. Indeed, few genes were found expressed in all species surveyed (for example out of nine genes, only Kallikrein was detected expressed in the mandibular salivary gland of all four species of varanid (Fry et al. 2006)). With increased taxon sampling, only Kallikrein and CRISP were detected in all 18 species of lizard sampled (Fry et al. 2010) which included 13 species of varanid. Whilst this may be an artefact of low sequencing depth, the lack of consistent expression should have precluded these genes being used to support a conserved repertoire of "venom" genes across the Toxicofera.

Perhaps the most significant issue with the evidence used to support the Toxocifera hypothesis is that all samples used for sequencing were derived from either salivary or venom glands, and no "body" tissues were included with which to compare gene expression. Transcriptomic analysis of solely venom gland is perfectly acceptable for descriptive studies which seek to characterise the transcriptome of this tissue. However, in order to assign a potential toxic role to a gene (and especially to infer its true evolutionary history, or the evolution of the venom repertoire in an entire lineage), sequencing the venom gland alone is insufficient. It has long been known that tissues all express a repertoire of "housekeeping" or maintenance genes (Butte et al. 2001) and as a result the sequencing of the entire venom or salivary gland will result in the identification of genes associated with a diverse range of functions (e.g. protein synthesis, cell-cell signalling and energy metabolism), not to mention that the sample will likely contain traces of other tissues such as muscle and blood. Consequently, genes cannot be inferred to encode toxins simply because they happen to be expressed in the venom or salivary gland.

Conservation in the structure of proteins detected in lizard oral secretions has also been used in support of the Toxicofera hypothesis. However, many secreted proteins, particularly members of the same gene family, have a conserved cysteine-rich "scaffold" (Anantharaman

et al. 2003). It should not be too surprising that related proteins have similar structures, especially as alterations to this scaffold, or to the conserved residues, would likely result in a disruption of the protein structure and function. Similarity of structure should not necessarily always be considered to reflect shared toxicity. When using the Australian snake venom detection kit, Jelinek et al. (Jelinek et al. 2004) found cross-reactivity between several snake species, most notably the tiger snake (*Notechis scutatus*) and the black-headed python (*Aspidites melanocephalus*). This has been used as evidence that putative toxin genes are translated into proteins in the venom or oral glands of these species, and that these proteins represent relics of an ancestral venom system which has been down-regulated in Henophidians (boas, pythons and several other families of basal snakes) (Fry et al. 2013). However, such cross-reactivity has been observed many years previously, with cross-reactivity demonstrated between colubrid oral secretions and antivenoms raised against African and Australian elapids (Minton and Weinstein 1987). Interestingly, the authors also found some antigenic cross-reactivity between a Henophidian snake (*Epicrates striatus strigilatus*) oral secretion when tested using a polyvalent antivenom raised against three *Dendroaspis* (mamba) species. Some of the responsible antigens were shown to be present in both venom and plasma, whilst some were present only in venom. Therefore, it is likely that some of this cross-reactivity between species is due to antigens present in secretions common to many species, as well as to cross-reaction between related members of protein families and cannot be taken as representative of any shared toxicity.

Whilst several Toxicofera-related studies commendably attempted to functionally test the oral secretions of some varanid lizard oral secretions, the results must be interpreted carefully. Purified group III PLA_2 from *V. varius* appears to have caused inhibition of platelet aggregation, although it is unclear why this was tested on human blood instead of the blood of native prey items such as birds or rabbits (Weavers 1989). It is also unclear as to whether

physiological concentrations (within a range of concentrations which occur naturally in oral secretions) of this protein were used in this assay or if an increased dosage was required to achieve this inhibition of platelet aggregation.

Crude mandibular oral secretion and synthesised natriuretic peptide from *V. varius* and *V. komodoensis* caused a drop in mean arterial pressure when injected intravenously into anaesthetised rats (Fry et al. 2006; Fry et al. 2009). However, intravenous (I.V.) administration is an unlikely delivery method in the event of a lizard bite, and the depressor effects of I.V. administration of saliva has been noted in previous experiments (Gibbs 1935; Levy and Appleton 1942). Therefore, physiological effects noted in a controlled laboratory experiment may not be translated in a real life scenario. For crude *V. varius* mandibular secretion, a concentration of 1mg kg^{-1} was required to cause a drop in blood pressure in an anaesthetised rat (Fry et al. 2006) whilst a decrease in blood pressure was seen at doses above 100µg/kg for synthesised natriuretic peptide (from *V. komodoensis*) with 400ug/kg required to induce hypotensive collapse (Fry et al. 2009). Conversely, in a similar experiment, 10µg/kg of crude Papuan taipan (*Oxyuranus scutellatus canni*) venom caused a complete respiratory and cardiovascular collapse (Crachi et al. 1999). It is safe to say that lizard "venom" is much more inefficient, and coupled with the inefficient delivery method in these species, is it realistic that they will administer sufficient amounts of toxin in a single bite?

Casting doubt on the Toxicofera hypothesis

The Toxicofera hypothesis has been widely accepted for almost a decade, and has proved to be pervasive and attractive. However, the downside of these qualities is that it has also avoided scrutiny and testing. There have recently been several studies which have cast doubt on the Toxicofera hypothesis (Hargreaves et al. 2014a; Reyes-Velasco et al. 2015), although their interpretation has led to alternative conclusions. Several phylogenetic analyses incorporating

non-venom gland transcriptomic data have shown that non-toxin sequences nest within clades of toxin genes, and it has been acknowledged that such findings provide "…strong evidence for the non-monophyly of Toxicoferan toxins" and that "…the results of [these] phylogenetic analyses would strongly refute the key prediction of the 'SEO' (single early origin) hypothesis…" (Casewell et al. 2012). Rather than accepting these conclusions, it has instead been proposed that venom gene recruitment may not be one-way, and that genes encoding venom toxins undergo a dynamic to-ing and fro-ing between toxin and physiological protein, whereby a venom toxin may undergo additional duplication, with subsequent recruitment back into a body tissue to fulfil a non-toxic physiological role. However, the more parsimonious hypothesis that these sequences actually represent *reptile* body sequences (which have never been toxins) forming *reptile* clades rather than body sequences nesting within *venom* clades is not considered. Similarly, Koludarov et al. (Koludarov et al. 2012) investigated the oral secretions of the lizard *Abronia graminea* and determined that "the NGF [nerve growth factor] expressed in venom may be the same gene as is used in the body and therefore may be a rare case of a venom protein resulting from a non-duplicated gene." It is possible that the product of a gene may be used pleiotropically as a toxin (fulfilling a toxic and non-toxic role simultaneously), but unless its expression is elevated in the salivary gland, there would be little evidence to suggest that it was anything more than a non-toxic physiological protein encoded by a housekeeping or maintenance gene.

More recent analyses incorporating an increased number of non-venom gland samples has further cast doubt on the Toxicofera hypothesis. A large scale test of the robustness of this hypothesis found that many of the genes used to support the single, early evolution of venom in squamates are in fact expressed in multiple body tissues including the salivary gland of a non-Toxicoferan lizard, the leopard gecko (*Eublepharis macularius*) (Hargreaves et al. 2014a). No evidence has been found of either a venom-specific splice variant or significantly elevated

expression level in the venom or salivary gland. Therefore, it is likely that these genes are simply encoding maintenance or "housekeeping" proteins, and are expressed in multiple tissues at low levels. Many of these genes were also found expressed in several other body tissues in *Echis coloratus* (Hargreaves et al. 2014b), adding further support that these are housekeeping genes due to their ubiquitous expression pattern. Several of these genes are also only present as a single copy in the genome of this species, and so there is no evidence of duplication and recruitment of a toxic version to the venom gland (Hargreaves et al. 2014a). Indeed, genes homologous to known toxins have been found expressed in the rictal gland, brain, intestine, kidney, testes, spleen, ovary, heart, stomach, liver, blood and muscle of the Burmese python (*Python molurus bivittatus*) and the venom gland, liver, pancreas, kidney, brain and heart of *Bothrops jararaca* (Junqueira-de-Azevedo et al. 2014; Reyes-Velasco et al. 2015). Whilst these results have been interpreted in different ways, they demonstrate that genes which are homologous to putative venom genes are expressed in many different tissues outside of the oral glands, and that sequencing solely the venom or salivary gland without other body tissues to use as a reference for gene expression is not enough. Interestingly, when the genome of the Burmese python was surveyed for genes orthologous to putative toxin genes, only one or two orthologs were detected for each toxin gene family. The authors suggest that the Burmese python is representative of the ancestral state, prior to the expansion of toxin gene families in the Caenophidia (Reyes-Velasco et al. 2015).

If the proteins encoded by these genes are not being used to fulfil a venomous function, why are they still being expressed in the oral secretions of these reptiles? Given the metabolic cost of producing venom (McCue 2006) it would be more logical that natural selection would act to end any unnecessary gene expression and protein synthesis. Indeed, this process has been shown to occur in the marbled sea snake, *Aipysurus eydouxii*, following a switch in diet from fish to sedentary fish eggs (Li et al. 2005a; Li et al. 2005b), whereby several toxin genes have

become pseudogenized (rendered non-functional via mutation). Why then has this not occurred in a plethora of reptile species which have no use for venomous function? Since many of the proposed toxins secreted by these glands are nothing of the sort, these oral secretions and the proteins they contain must have alternative functions, incorporating aspects of lubrication, pre-digestion and the stimulation of digestive processes and anti-microbial activity (Weinstein et al. 2012).

Glands and fangs

Reptiles possess many salivary glands that secrete into the oral cavity, with a key role in the lubrication of food. Many are mucous in nature, however, some glands also have serous secretions which, in some cases, have become adapted as venom producing glands, as observed in venomous (Helodermatid) lizards, front-fanged snakes and some rear-fanged snakes.

In front-fanged snakes (such as elapids and vipers) and rear–fanged snakes, the fang and venom gland develop from a region at the back of the maxillary dental lamina (Vonk et al. 2008). The final position of the fangs is therefore attained by movement of the growing fangs, forward or backwards in the mouth, after initiation. Importantly in these venomous snakes, the venom gland and the fang appear to form from a united primordium that starts as an epithelial thickening below the eye on the upper jaw. This thickening has been called the primitive dental ridge (Martin 1899). In *Vipera palaestinae*, the thickening splits into an anterior gland and more posterior fang, with the venom gland extending first anteriorly before turning posteriorly and branching (Kochva 1963). In contrast to the serous venom gland, the nearby supralabial glands develop from independent placodes and are generally mucous.

In the rear-fanged snakes (Colubridae) the fang is associated with the Duvernoy's gland, which appears not to act as a venom gland and has instead been proposed to have an anti-bacterial role in coating dental surfaces (Jansen 1983). Secretion from the Duvernoy's gland in *Thamnophis elegans vagrans* was found to have enhanced anti-bacterial properties when

compared to supralabial glands (Jansen 1983). In addition to a similar position of the fang primordium when compared to front-fanged snakes, the fang and venom gland of rear-fanged snakes also develops from a united primordium, as has been described in the opisthoglyph *Telescopus fallax* and aglyph *Thamnophis sirtalis* (Kochva 1965). *Telescopus* has a complete row of maxillary teeth with the fang primordia and gland forming at the posterior end. In contrast to the viperidae the venom gland does not first grow anteriorly before growing posteriorly. The fact that in these different snakes the venom gland and fang initiate from a common primordium that forms at the back of the maxillary dental lamina indicates that these front and rear fangs are homologous structures (see also (Vonk et al. 2008)). Importantly, Duvernoy's glands do not appear to form at all in many colubrids, for example some species of the genus *Elaphe*, genera *Lampropeltis*, *Pituophis*, *Pseuetes*, *Rhinocheilus* and *Spilotes* (Taub 1967). A variety of *Elaphe* species used in this study (although some of these have since been assigned to different genera) have no Duvernoy's gland and their supralabial glands are purely mucous (Taub 1967). In general such snakes without a Duvernoy's gland are constrictors who suffocate their prey before digestion. The lack of large serous glands in these species has been suggested to be due to secondary loss (Underwood and Kochva 1993; Vidal 2002). Although this may well be correct in some derived forms it is also possible that the Duvernoy's gland may not have evolved in all snakes, indicating independent evolution of this gland. Supporting this idea, Boidae and other primitive snakes have mainly mucous salivary glands, which are found at a range of positions in the oral cavity (Kochva and Gans 1970) In Boidae, anterior temporal glands composed of serous cells have been described at the back of the maxilla (Taub 1966). Supralabial glands are generally thought of as mucous in most snakes but some Colubrids have serous cells included in the supralabial glands (Taub 1967). Thus whether a gland is mucous or serous is subject to some variation across reptiles and, in keeping with this, Duvenoy's glands can be mucous in part in some Colubridae (Taub 1967). Whether

a gland is serous or mucous, therefore, cannot be necessarily used to infer evolutionary relationships.

In both front and rear fanged snakes, the fangs are associated with a gland that forms from the same dental primordium as the tooth. These are therefore true dental glands. Any homologous structures would therefore be proposed to share this joint origin. It is therefore important to know whether venom glands in Toxicoferan lizards also develop from a united dental placode. If not, they are unlikely to be homologous, but instead would represent independent adaptations to venom formation in other oral glands. Some oral glands in lizards do indeed appear to develop from a lamina linked to the dental lamina. For example in chameleons the tooth and dental gland appear to share a similar origin (Tucker 2010). However in helodermatids, where venom glands are found on the lower jaw, the glands lie adjacent to the tooth with the duct at a slight distance when viewed in section (Kardong et al. 2009), indicating that the tooth and gland develop from separate placodes. Supporting this view, the ducts have been proposed to run to an opening between the lip and the jaw, rather than to the base of the teeth (Shufeldt 1891) and the location of the gland appears more similar to an infralabial gland. From MRI, however, the gland ducts of helodermids appear to terminate at the base of teeth (Fry et al., 2010), suggesting a closer relationship with the dentition. Further understanding of the anatomy and development of the venom glands of helodermatids is important to be able to ascertain whether they are homologous to those of snakes.

The lack of a developmental link between dental glands and teeth in venomous lizards compared to snakes, and the lack of a large serous gland associated with the maxillary dental lamina in primitive snakes and some colubrids strongly suggests that the venom delivery system in snakes and lizards evolved independently. From the presence of Duvernoy's glands in snakes without venom, it would appear that the Duvernoy's gland first evolved as a branch of the forming dental lamina and then was adapted into a venom-producing gland in both front

401 and rear-fanged snakes. A clear understanding of the embryonic development of the venom
402 glands in venomous lizards will be important to clarify such points.

403 **Varanid venom**

404 Many Toxicofera-related studies suggest that lizards belonging to the genus *Varanus* are in fact
405 venomous, in particular the Komodo dragon *V. komodoensis* (Fry et al. 2006; Fry et al. 2009;
406 Fry et al. 2013). A review of the available evidence found it unlikely that the Komodo dragon
407 utilises venom as a prey capture method, instead suggesting that if it did use venom it was used
408 as a pre-digestion method (Arbuckle 2009). Historical field observations have suggested that
409 blood loss due to injury is the main prey capture strategy utilised by Komodo dragons
410 (Auffenberg 1981). Whilst many *Varanus* species have been kept in captivity for many years,
411 there have been almost no reports of any symptoms concurrent with envenomation following
412 a bite. In the original Toxicofera paper (Fry et al. 2006) there are anecdotal reports of bites
413 from three species of *Varanus* which resulted in symptoms such as dizziness and rapid
414 swelling. Most recently, a bite by a Bengal monitor (*Varanus bengalensis*) reportedly caused
415 acute kidney injury to a human patient, which ultimately (and most unfortunately) resulted in
416 death (Vikrant and Verma 2014). However, no positive identification was made of the
417 offending animal, other than the name given by the patient. Perhaps more dubious is that the
418 bite symptoms were more in line with envenoming from a Russell's viper (*Daboia russeli*)
419 (White and Weinstein 2015), a member of the so-called "Big four" and a main cause of
420 mortality due to snakebite in India (Simpson and Norris 2007). Unfortunately no mention is
421 made of the bite wound itself which may aid in distinguishing between a lizard or snake as the
422 culprit. Additionally, a recent bite by a Komodo dragon reportedly resulted in no symptoms of
423 envenomation (Borek and Charlton 2015). Therefore, the status of varanid lizards as venomous
424 is uncertain, particularly when compared to known venomous lizards such as the Gila monster
425 and beaded lizards.

Conclusions and future directions

Venom evolved multiple times in reptile evolution

Whilst the Toxicofera hypothesis represents a parsimonious explanation of the evolution of venom in reptiles (one character evolving a single time), the inclusion of non-venom-gland derived transcriptomic data in phylogenetic analyses along with the quantification of gene expression would strongly suggest that the Toxicofera hypothesis is unsupported (Hargreaves et al. 2014a). This would prompt a move back to the previous hypothesis that venom has evolved multiple times within squamate reptiles, once in the advanced snakes, once in the helodermatid lizards, and potentially another time in varanid lizards (although more evidence is needed to confirm this). This is in keeping with the large phylogenetic distance between venomous snakes and venomous lizards, the differing morphology of venom delivery systems between these animals (e.g. gland location, teeth/fangs), and the differing uses for their venoms (i.e. snakes predominantly for prey capture and helodermatid lizards for defence).

Simplified complexity of reptile venom

The rejection of the Toxicofera hypothesis and the ruling out of many of the genes used to support it as toxins leads to an inescapable conclusion, that snake venom is not as complex as previously suggested (Li et al. 2005b; Kini and Doley 2010; Casewell et al. 2013). A review of venom proteome data from several species (Calvete et al. 2007; Wagstaff et al. 2009; Vonk et al. 2013) shows that snake venom is composed of a relatively small number of gene families encoding a few dozen different proteins, with most extensive diversity found in only one or a few of these families (Calvete 2013; Hargreaves et al. 2014a). Whilst post-translational

451 modifications may prove to play a significant role in generating more extensive diversity from
452 a limited genetic background (Casewell et al. 2014), the idea that snake venom is a "complex
453 cocktail" (Casewell et al. 2013) of hundreds of different proteins encoded by many gene
454 families seems to be unsupported by experimental evidence. The low number of products in
455 snake venom makes perfect sense as (1) a complex proteinaceous mixture would be
456 metabolically expensive to produce and (2) natural selection will act to streamline the venom,
457 tailoring it to the snakes' prey items. In short, a simple venom is efficient; a complex venom is
458 overkill. The implications of this reduced complexity are significant, particularly for the
459 development of the next generation of antivenom treatments utilising methods such as "string
460 of beads" (Whitton et al. 1993) and "epitope-string" (Casewell et al. 2013). A reduction in the
461 number of likely toxins inherently means a reduction in the number of targets requiring
462 neutralisation by antivenom, and as a consequence the reduced number of components
463 contained in the antivenom would mean a reduction in antigenicity, meaning a reduced chance
464 of adverse reactions to treatment such as anaphylaxis and serum sickness (Nuchprayoon and
465 Garner 1999).
466 From an evolutionary perspective, the reduction in the number of toxins does not detract from
467 the fascination or specialization of venoms, in fact the opposite is true. The occurrence of
468 lineage-specific gene duplications (for example *complement c3* and *nerve growth factor* in
469 Elapids (Sunagar et al. 2013; Hargreaves et al. 2014a; Hargreaves et al. 2014b)) would indicate
470 that these genes may confer some prey-specific effects (as seen in the Mangrove catsnake,
471 *Boiga dendrophilia* (Pawlak et al. 2006)), or may have allowed adaptation to a new ecological
472 niche.
473
474 *The changing definition of venom*

The Oxford English dictionary defines venom as "a poisonous substance secreted by animals such as snakes, spiders, and scorpions and typically injected into prey or aggressors by biting and stinging". A more specific and long-standing definition would be "a complex substance produced in a specialized gland and delivered by an associated specialized apparatus that is deleterious to other organisms in a given dosage and is actively used in the subjugation and/or digestion of prey and/or in defence" (Mebs 2002). More recently, the quest for a catch-all term that encompasses the diverse uses of venom by insects, molluscs, reptiles and mammals has led to increasingly broad definitions of venom, such as "a secretion, produced in a specialised tissue (generally encapsulated in a gland) in one animal and delivered to a target animal through the infliction of a wound (regardless of how tiny it is). A venom must further contain molecules that disrupt normal physiological or biochemical processes so as to facilitate feeding or defence by/of the producing animal" (Fry et al. 2012b). It is perhaps time to discard this quest in favour of more restricted, possibly even lineage-specific, terminology with emphasis on the biological role of the venom to the survival of the animal. As an example, human saliva contains many of the proteins encoded by the same gene families which are also found present in the snake venom proteome, including cystatins, disintegrin-like metalloproteinases, epididymal secretory protein E1, group IIA PLA_2s, β-defensins, and kallikrein (Hu et al. 2005; Guo et al. 2006). Human saliva has also been shown to be toxic (Bonilla et al. 1971). However, humans are not considered to be venomous, we do not use these secretions to kill or otherwise incapacitate prey, and so these proteins must fulfil some other biological role, such as pre-digestion and lubrication. Therefore, the presence of proteins homologous to known (or proposed) toxin proteins in oral secretions does not automatically mean that the organism is venomous. Moreover, considering the presence of homologous proteins in the oral secretions of basal snakes as toxins based on their use as toxins in more derived species, without evidence of these proteins showing any functional significance, is an erroneous and premature

assumption, which has been stated previously by other authors (Kardong 2012; Weinstein et al. 2012).

Future directions

The increased application of second generation DNA sequencing technologies and the integration of multiple types of 'omic data (genomic, transcriptomic, proteomic) is revolutionising the study of the evolution and composition of venom in reptiles, with implications not only for our understanding of this evolutionary innovation, but also for the treatment of snakebite and development of novel pharmaceuticals. Once the genome to proteome path of toxin expression is completely elucidated, this leaves the fundamentally important question: what do these proteins actually do? Perhaps more pertinent, is the functional property of these proteins relevant to the biological role of the venom and to the survival of the animal? Oral secretions are likely to have several biological roles, such as pre-digestion and lubrication, and so some proteins are likely to fulfil these rather than act as venom toxins. Only with functional characterisation (which can be a long and arduous task, particularly compared to the "one-shot" nature of high throughput sequencing) of these putative toxins can a true role be assigned to them. Moreover, functional testing of proteins should be performed at physiological concentrations on native prey items.

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