The evolution of fangs, venom and mimicry systems in blenny fishes

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Summary

Venom systems have evolved on multiple occasions across the animal kingdom and they can act as key adaptations to protect animals from predators [1]. Consequently, venomous animals serve as models for a rich source of mimicry types, as non-venomous species benefit from reductions in predation risk by mimicking the coloration, body shape and/or movement of toxic counterparts [2–5]. The frequent evolution of such deceitful imitations provides notable examples of phenotypic convergence, and are often invoked as classic exemplars of evolution by natural selection. Here, we investigate the evolution of fangs, venom and mimetic relationships in reef fishes from the tribe Nemophini (fangblennies). Comparative morphological analyses reveal that enlarged canine teeth (fangs) originated at the base of the Nemophini radiation, and have enabled a micropredatory feeding strategy in non-venomous *Plagiotremus* spp. Subsequently, the evolution of deep anterior grooves and their coupling to venom secretory tissue provides *Meiacanthus* spp. with toxic venom they effectively employ for defense. We find that fangblenny venom contains a number of toxic components that have been independently recruited into other animal venoms, some of which cause toxicity via interactions with opioid receptors, and result in a multifunctional biochemical phenotype that exerts potent hypotensive effects. The evolution of fangblenny venom has seemingly led to phenotypic convergence via the formation of a diverse array of mimetic relationships that provide protective (Batesian mimicry) and predatory (aggressive mimicry) benefits to other fishes [2,6]. Our results further our understanding of how novel morphological and biochemical adaptations stimulate ecological interactions in the natural world.

Results and discussion

Fishes of the tribe Nemophini, known as fangblennies, represent a unique system for studying the adaptations underpinning the formation of mimetic relationships. This tribe consists of five genera: the venomous genus *Meiacanthus* and four non-venomous genera, of which *Plagiotremus* and *Petroscirtes* contain species that mimic the aposematic coloration and behavior of *Meiacanthus* [2,6,7] (Figure 1A). A number of fangblenny models are mimicked by multiple sympatric fish species (see [8] for a thorough overview), and while Batesian mimicry prevails in all such relationships, some members of the genus *Plagiotremus* also use mimicry in an aggressive manner, to gain access to larger fishes to feed on their scales and fins [2,6] (Figure 1A). Whilst other fangblennies (e.g. *Aspidontus taeniatus*) are known to mimic non-*Meiacanthus* models, such as the cleaner wrasse *Labroides dimidiatus* [2], for the purposes of this study we focus only on mimetic relationships in which venomous *Meiacanthus* fangblennies are the models.

We first reconstructed the evolutionary relationship of fangblennies by sequencing five molecular markers from representative Nemophini species (Table S1). Our concatenated dataset (n=36; 2,691 bp) produces a strongly supported tree topology (Figure S1) largely consistent with that of Hundt et al. [9]. However, in our tree the venomous genus *Meiacanthus* forms a strongly supported sister clade to a monophyletic group containing the genera *Aspidontus* and *Petroscirtes* (Figure 1B), whereas in Hundt et al. [9] *Meiacanthus* was found sister to *Plagiotremus* and *Xiphasia* without strong support. In our analysis, the remaining genera, *Xiphasia* and *Plagiotremus*,

form a monophyletic group sister to that of *Meiacanthus*, *Aspidontus* and *Petroscirtes*.

We next used micro-computer tomography (microCT) scanning, stacking microscopy and histology to provide a comprehensive overview of the oral morphology of fangblennies and their close relatives. Our comparative morphological analyses demonstrate that all fangblennies have enlarged canine teeth (fangs) on their lower jaw and buccal epithelium surface areas in comparison with their relatives (Figure 2, Figure 3A and 3B and Figures S1 and S2). Histological analyses reveal that all members of the Nemophini have hollow fangs, and whilst *Meiacanthus* and *Petroscirtes* both have a maxillary sheath to accommodate the enlarged teeth, only *Meiacanthus* spp. possess anterior grooves for the transmission of venom. Similarly, only *Meiacanthus* spp. have venom glands (Figure 2 and Figure 3C and 3D). Three-dimensional reconstructions of histological sections of *M. grammistes* and *M. reticulatus* venom glands show that they surround the base of the fangs posteriorly and enter the anterior groove, an arrangement which presumably facilitates the transmission of venom from the venom gland into the target animal during biting (Figure 3E and 3F).

Overlaying the presence or absence of (i) enlarged canine teeth and (ii) venom glands onto the species phylogeny revealed a single most parsimonious explanation for the origin of each of these characters, namely: the combined presence of enlarged canines at the base of the tribe Nemophini, and venom glands at the base of the *Meiacanthus* radiation

(Figure 1B). Therefore, unlike venomous snakes where the chemical weapon preceded the refined venom delivery dentition [10], fangblennies evolved mechanical structures amenable for venom delivery prior to the origin of their toxic secretions.

Little is known about the fangblenny venom system, other than enlarged canine teeth deliver venom into aggressors to prevent ingestion [11–14]. The defensive nature of the venom is perhaps best evidenced by observations of multiple predatory fishes ingesting *M. atrodorsalis* before “quivering of the head with distention of the jaws and operculi” occurred, followed by the fangblenny emerging from the mouth unharmed [13]. Furthermore, feeding experiments with *M. atrodorsalis* demonstrated that when the canine fangs were removed, predatory fish readily consumed the fangblenny, but with fangs intact they were expelled and avoided in subsequent encounters [13].

The oral venom system of *Meiacanthus* is exceptional among teleosts, as venom is typically delivered via the mechanical rupture of secretory cells associated with dorsal and/or opercular spines [15]. Indeed, the use of an oral venom system exclusively for defensive purposes is unusual in the animal kingdom. We suggest that the absence of large fin spines amenable for effective venom delivery in blenny ancestors, coupled with the enlargement of canine fangs, has facilitated the evolution of oral venom observed in *Meiacanthus*. Eels of the genus *Monognathus* are the only other fishes thought to have a venomous bite [15], although they are thought to primarily use their venom for predatory purposes. Nonetheless, we note that ancestors

to these fishes also lack large fin spines suitable for defensive purposes [16], suggesting an element of constraint.

To investigate the toxin composition of fangblenny venom we constructed transcriptomes from the venom gland of *M. atrodorsalis* and tissue from the corresponding location in the non-venomous species *Plagiotremus tapeinosoma*, and we performed proteomic analyses on venom extracted from *M. grammistes* (Figure 4A). Putative toxins were identified by their abundant expression in the venom gland transcriptome and their absence, or low-level expression, in the control transcriptome, coupled with their detection in secreted venom. Whilst many of the proteomic matches were to genes encoding constitutive housekeeping proteins, we found three toxin types, none of which have previously been reported from fish venom, that exhibit characteristics consistent with venom-specific roles: group X phospholipases A_2 (PLA_2), proenkephalin and neuropeptide Y (Figure S3).

Proenkephalin and neuropeptide Y were both found expressed in the *M. atrodorsalis* venom gland transcriptome, identified proteomically in *M. grammistes* venom and were completely absent from the *P. tapeinosoma* control transcriptome, strongly suggesting venom-specific roles. Although genes encoding group X PLA_2s were detected in both transcriptomes, the expression level observed in the *P. tapeinosoma* control transcriptome was extremely low (0.04%). In contrast, both PLA_2 and neuropeptide Y were heavily expressed in the venom gland transcriptome, with single contigs representing the third and fourth most abundant annotated contigs (1.30%

and 1.00% respectively – Table S2), while proenkephalin exhibited a more moderate expression level (0.15%; 69th most abundant).

Secreted PLA_2s hydrolyse ester bonds of glycerophospholipids to produce fatty acids and lysophospholipids, and they are common constituents in animal venoms (e.g. bees, scorpions, snakes [17]). While the group X class of PLA_2s has not previously been described from any venom, they are known to promote inflammatory pathology [18,19]. Using a fluorescent *in vitro* enzyme assay we demonstrated that fangblenny venom exhibits considerable PLA_2 activity and causes dose-dependent cleavage of a PLA_2-sepecific substrate (Figure 4B). To put these results into biological context, we compared the PLA_2 activity of fangblenny venom with those of two viperid snakes (*Tropidolaemus wagleri* and *Parias hageni*) known to have venom PLA_2s [20,21]. We find comparable levels of substrate-cleavage between the different venoms (Figure 4B), suggesting that fangblenny PLA_2 is likely a biologically relevant venom toxin.

Proenkephalin encodes multiple five amino acid peptides known as met-enkephalins, which are endogenous opioid hormones that function by interacting with opioid receptors and induce transient analgesia, hypotension and inflammatory responses [22–24]. To test for opioid activity, we screened fangblenny venom against human embryonic kidney 293 (HEK) cells expressing μ-, κ- and δ-subtype opioid receptors. The δ and μ, but not κ, displayed significant inhibition of cAMP production in the presence of fangblenny venom, with the greatest reduction seen with the δ cell line (Figure

4C and Figure S4). To confirm that the inhibition of cAMP observed in the δ and μ cells was mediated through the opioid receptors, naloxone, a non-selective opioid receptor antagonist, was used to block receptor activity. We find that the inhibited production of cAMP caused by fangblenny venom was largely blocked by naloxone in cells expressing δ-subtype opioid receptors, but not in those expressing μ (Figure 4D and Figure S4). These results demonstrate that, in a similar manner to those identified from the venom of the scorpion *B. martensii* [25], enkephalin peptides found in *Meiacanthus* induce physiological effects via their interaction with δ-subtype opioid receptors.

Neuropeptide Y provides another example of the same starting substrate being convergently utilized for a role in animal venom, having previously been identified in the cone snail *Conus betulinus* [26]. These peptides are relatively well-conserved, are found widely distributed in nervous systems and are crucial for the regulation of cardiovascular processes such as blood pressure [27]. Consequently, we assessed the bioactivity of fangblenny venom in *in vivo* cardiovascular assays. We found that *M. grammistes* venom caused a marked depressor effect on the mean arterial pressure of the anaesthetized rat (Figure 4E), consisting of a transient depressor response followed by a sustained depressor response and resulting in a maximal decrease of 37% (±5%). Despite this potent hypotensive bioactivity, we found that *M. grammistes* venom had no significant effect on the heart rate of the anaesthetized rat (Figure 4F). These results are highly suggestive in regards to neuropeptide Y and enkephalins; both peptides, detected here in

fangblenny venom, have previously been demonstrated to significantly reduce blood pressure *in vivo*, without having any discernible effect on heart rate [23,28].

Given prior reports of some fish venoms exhibiting neuronal bioactivity [29], we next tested the neurotoxic effect of fangblenny venom in the chick biventer cervicis nerve-muscle (CBCNM) preparation. *M. grammistes* venom exhibited a weak neurotoxic effect by causing a significant decrease in indirect twitches of the CBCNM over 60 min (Figure 4G), but did not inhibit responses to exogenous acetylcholine, carbachol or potassium chloride, thereby indicating a lack of activity at skeletal muscle nicotinic receptors (Figure 4H). It remains unclear which component(s) in fangblenny venom is responsible for causing this neurotoxic bioactivity, although we note that some PLA_2s found in snake venom have previously been described to cause neurotoxicity [30,31].

Spine-delivered fish venoms are typically notoriously painful and the primary pathology observed following envenomings is pain disproportionate to the wound [29,32]. Considering such fish use their venom for defensive purposes, pain is an effective tool for deterring predators and invoking learned avoidance responses. Consequently, the use of pain-inducing molecules has evolved convergently in many other venomous lineages that use venom for defensive purposes [1,33]. However, when we sub-cutaneously injected fangblenny venom into the hind paw of the anaesthetized mouse we observed no evidence of behavioral characteristics consistent with pain (paw lifts, licks, shakes and flinches) and no difference between envenomed and control

animals. These data correlate with some reports of human bites by fangblennies being relatively painless [13]. Therefore, in contrast to the spine-delivered venom employed by most venomous fish, we find that the oral venom of the fangblenny does not induce immediate, substantial, pain to mammals. While species-specific nociceptive effects are possible, our data suggest that this defensive venom is surprisingly multi-functional, being markedly hypotensive (via neuropeptide Y and/or enkephalins), weakly neurotoxic (unknown components/possibly PLA_2s) and perhaps also proinflammatory (PLA_2s and/or enkephalins). The combination of these venom bioactivities therefore appears sufficient to effectively confer distastefulness and learned avoidance behaviors in piscine predators [13], perhaps irrespective of any potent nociceptive effect. Indeed, the pronounced hypotensive effects induced by venom peptides seem highly likely to affect the co-ordination and/or swim performance of envenomed fishes, and therefore likely confer a fitness advantage to the fangblenny by facilitating escape from predators.

The evolution of venom in *Meiacanthus* fangblennies appears likely to have been a contributing factor to many other non-venomous fish coevolving similar aposematic color patterns and swimming behaviors, thus becoming Batesian mimics and benefiting from reduced predation pressures [2,6,7,34]. These putative mimics include other fangblennies (e.g. *Petroscirtes breviceps* and *Plagiotremus* spp.) and a variety of other distantly related fish (e.g. the combtooth blenny *Escenius gravieri* and the cardinalfish *Cheliodipterus nigrotaeniatus*) (Figure 1A). Moreover, the evolution of enlarged fangs in the

tribe Nemophini appears to have also stimulated a unique micropredatory feeding strategy in the genus *Plagiotremus* as, to our knowledge, all species in this genus feed by attacking larger reef fishes to access dermal tissue, scales, mucus and fins [35,36]. For a number of species micropredation is facilitated by resembling venomous *Meiacanthus* fangblennies – mimicry provides increased access to these resources, and thus interactions between *Meiacanthus* and *Plagiotremus* represent one of the few described examples of Batesian-aggressive mimicry [2,6].

In summary, venomous animals provide some of the most striking examples of functional convergence, relating to their diverse, yet often similar, biochemical phenotypes [1]. In addition, they serve as models for a rich source of mimicry types that span the full range of mimetic relationships: from Batesian (e.g. coral snakes [5]), to Müllerian (e.g. neotropical catfish [3]) to aggressive (e.g. fangblennies [6]). Herein we characterized the venom system of *Meiacanthus* fangblennies to understand how the evolution of toxicity has facilitated the evolution of novel mimicry types and, consequently, has stimulated a variety of mimetic interactions with a diverse array of other fishes via the process of convergence. Revealing the toxic basis of these classical vertebrate mimicry models furthers our understanding of how genotypic and morphological adaptations result in phenotypic novelty, which in turn stimulate new ecological interactions in the natural world.

Experimental procedures

A full description of the experimental procedures can be found in the Supplemental Information.

Phylogenetic reconstruction

We extracted genomic DNA from 36 specimens of 11 species of blenny (Table S1) and used a PCR and Sanger sequencing approach to sequence two mitochondrial (12S and 16S) and two nuclear (MYH6 and PTR) markers. The resulting sequence data was aligned, concatenated into a single partitioned dataset (n=36; 2,691 bp) and a species tree reconstructed using Bayesian inference [37] ($10x10^6$ generations) with optimized models of sequence evolution implemented (GTR+G for mitochondrial genes and HKY+G for nuclear genes). DNA sequence data has been submitted to the nucleotide database of GenBank (KY020158-KY020235).

Imaging and histology

We scanned representative blennies (Table S1) with micro-CT (Skyscan 1076) at 9 µm and 16.6 µm (*M. grammistes* cranial reconstructions) resolution and reconstructed the scans in 3D using ImageJ v1.51f, Materialise Mimics v19.0 and Meshlab v1.3.3. The lower jaws of one specimen per species were also dissected and analyzed with a Zeiss stacking microscope and photographs taken using an AxioCam MRc5 (Zeiss). For histology, dissected blenny heads were first decalcified and processed for paraffin histology using Histoclear as the intermediate reagent. Heads were serially sectioned at 7 µm (transverse), stained with Mayer's Haematoxylin, 1% eosin and 1% Alcian

blue in 2.5% acetic acid. Three-dimensional (3D) reconstructions were made in Amira version 5.3.3 (FEI Visualization Sciences Group, Bordeaux).

Transcriptomics

Venom glands and corresponding lower jaw tissue were dissected and pooled from ten specimens of *M. atrodorsalis* and *P. tapeinosoma* respectively. We generated transcriptomes as previously described [38] and assembled the resulting 2.56 (*M. atrodorsalis*) and 3.08 (*P. tapeinosoma*) million 250 bp paired-end reads using Trinity v2.1.1. Raw sequence data has been submitted to the sequence read archive (SRA) database of GenBank with the BioProject number PRJNA347283. The assembled transcriptome contigs are available from Mendeley Data (http://dx.doi.org/10.17632/cj2x496wp4.1).

Proteomics

We extracted venom from the fangblenny *M. grammistes* and characterized the protein profile using one dimensional SDS-PAGE gel electrophoresis under reducing conditions with 20 µg of venom. To identify proteins present in venom we used a shotgun sequencing approach that we previously validated [38]. Resulting mass spectra were analyzed with ProteinPilot V4.0 (ABSciex, USA) and peptides identified via BLAST searching the UniProt database and our translated transcriptome databases. An excel datasheet detailing the proteomic data and annotations is available from Mendeley Data (http://dx.doi.org/10.17632/cj2x496wp4.1).

Bioactivity

All animal experimentation was undertaken with approval from the University of Queensland (B.G.F., I.V.), Melbourne University (B.G.F.) and Monash University (W.C.H.) animal ethics committees. We tested for continuous PLA_2 enzymatic activity in venom (0.5 and 1.0 µg) using the EnzChek® Phospholipase A_2 Assay Kit protocol (ThermoFisher Scientific) and triplicate measurements over 100 cycles. A cAMP alphascreen assay was used to determine the activity of fangblenny venom (10.0, 1.0 and 0.1 µg/ml) on opioid receptors (δ, µ and κ) and was performed as previously described [39], by stimulating with forskolin (80 µM) and with and without naloxone (50 µM) present. We tested the pain-inducing activity of fangblenny venom by subcutaneously injecting 20 µg of venom (1 µg/µl saline solution) into the left hind paw of anaesthetized mice (n=3) and monitoring pain behaviors (paw lifts, licks, shakes and flinches) for 15 minutes in comparison with control animals injected with saline. The effect of venom (50 µg protein/kg) on blood pressure and heart rate was examined in anaesthetized rats (n=3), as described previously [40]. Responses to venom were expressed as percentage changes from the pre-venom baseline. Neurotoxic venom effects were examined using the previously described CBCNM preparation [31,40] and we monitored the effect of venom (2.5 µg/ml) on indirect twitches (n=4) and responses to exogenous acetylcholine (ACh; 1 mM), carbachol (CCh; 20 µM) and potassium chloride (KCl; 40mM). Responses were expressed as percentages of pre-venom addition.

Author Contributions

N.R.C and B.G.F. designed the research. N.R.C., G.M.C., K.L.C. and B.G.F. collected samples. N.R.C. and G.C.B. constructed the species tree. J.C.V., A.R., V.W., I.Q., L.v.d.W., M.K.R. and B.G.F. performed morphology work. N.R.C., S.C.W. and B.G.F constructed the transcriptomes. J.C.V., K.B., S.A.A., J.Do., A.N. and B.G.F. performed proteomic experiments. N.R.C., K.B. and B.G.F. analysed the gene and protein data. H.H., S.K., M.M., J.De., I.K., W.H., and I.V. performed bioactivity studies. N.R.C. wrote the manuscript with assistance from B.G.F. and input from all other authors.

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Supplemental Information

The Supplemental Information contains the supplemental experimental procedures, four figures, two tables and the supplemental references.

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Figures and legends

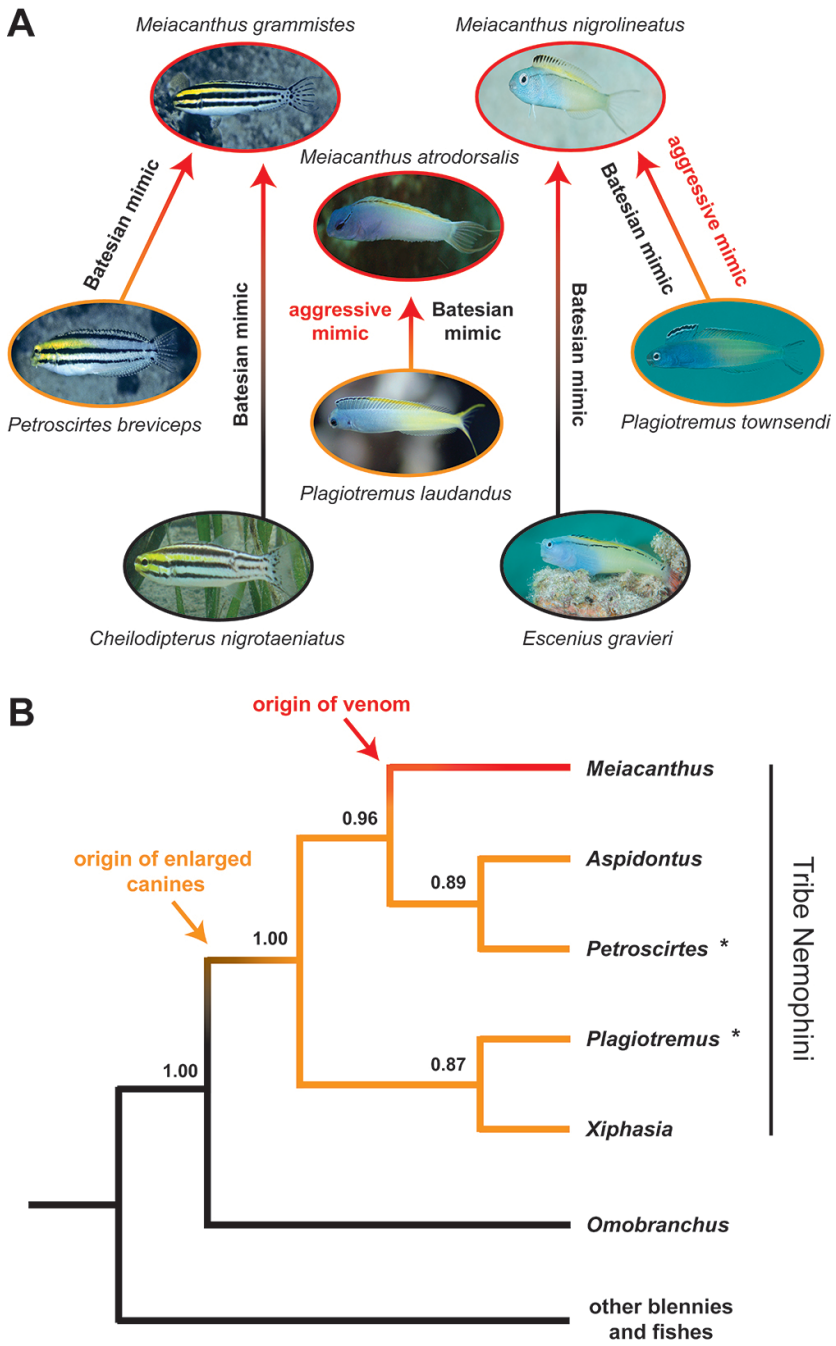

Figure 1. Examples of mimetic relationships involving *Meiacanthus* and the phylogenetic relationship of fangblennies. A) Examples of venomous *Meiacanthus* fangblennies (red circles) serving as models in mimetic relationships with other non-venomous fangblennies (orange circles) and non-fangblenny species (black circles). These relationships include Batesian and aggressive mimicry [2,6,7]. **B)** Schematic topology of the relationship between

different genera found in the Tribe Nemophini (see also Figure S1 and Table S1) and the single most parsimonious timings for the origin of enlarged canine teeth (fangs) and venom. Numbers at nodes represent Bayesian posterior probabilities and asterisks indicate genera that contain at least one member known to mimic *Meiacanthus* fangblennies [2]. Photos courtesy of Rudie Kuiter, Arthur Bos, Richard Smith (oceanrealimages.com) and Karen Cheney.

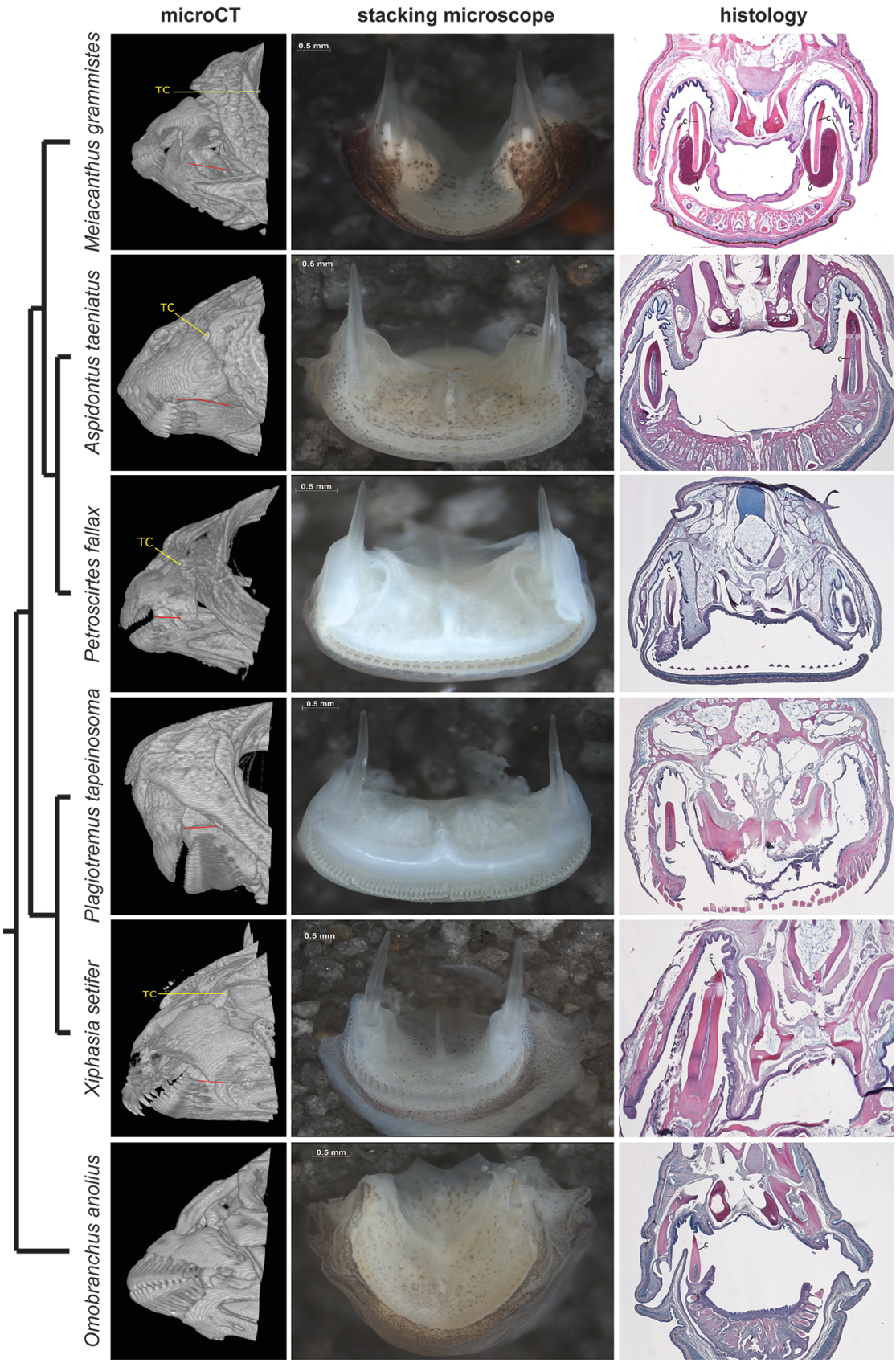

Figure 2. Oral morphology of the canines and venom system of fangblennies (Tribe Nemophini). Left panel; lateral view of micro-CT scans.

Red lines indicate the base of enlarged canines and yellow lines labelled TC indicate the tip of the canine. Middle panel; rostral view of the lower jaw by stacking microscope. Right panel; histology sections showing the oral cavity at 2x zoom. Annotations: C – canine, V – venom gland (*Meiacanthus grammistes* only). Note the smaller comparative fang size in the outgroup species *Omobranchus anolius* (Tribe Omobranchiini). See also Figure S2.

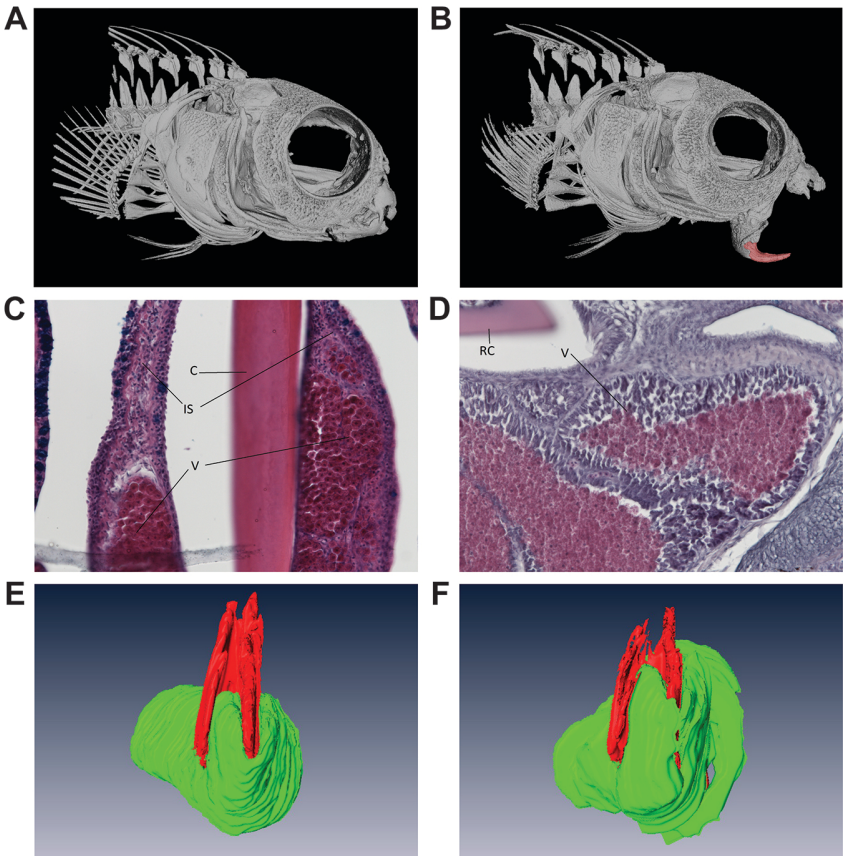

Figure 3. Morphology of the *Meiacanthus* venom system. A) and **B)** Lateral view of micro-CT scans of *M. grammistes* showing the size of the enlarged venom-transmitting fangs (coloured red) in mouth closed and mouth open positions, respectively (see also Figure S1). **C)** 20x zoomed histological section of *M. grammistes* showing the anterior region of the venom gland with deep purple cells, a canine tooth and enveloping connective tissue. **D)** 20x zoomed histological section of *M. reticulatus* showing a depleted venom gland (posterior portion). Annotations: C – canine; V – venom gland; IS – integumentary sheath; RC – replacement canine. **E)** and **F)** 3D reconstructions of histological sections from *M. grammistes* and *M. reticulatus*, respectively, showing the venom glands (green) surrounding the base of the canine tooth (red) and entering the anterior groove of the canine. Note that the canine reconstructions are incomplete.

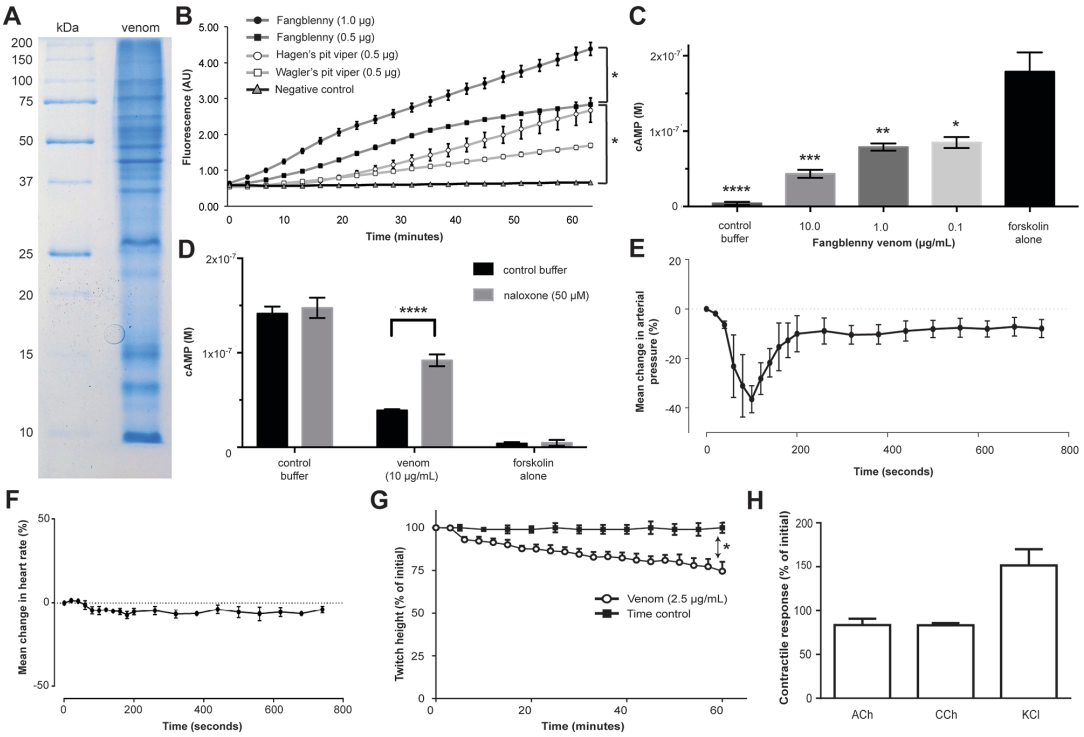

Figure 4. The bioactivity of venom from the fangblenny *Meiacanthus grammistes*. A) Reduced SDS-PAGE gel electrophoretic profile of extracted venom. **B)** Fangblenny venom (0.5 µg and 1.0 µg) exhibits dose-dependent phospholipase activity via the cleavage of a PLA_2-specific fluorescent substrate. Venom PLA_2 activity is comparable to those determined from the snakes *Parias hageni* (Hagen's pit viper) and *Tropidolaemus wagleri* (Wagler's pit viper). * indicates significant differences ($P \leq 0.01$; unpaired *t*-test). See also Figure S3 and Table S2 for information on fangblenny PLA_2. **C)** Fangblenny venom (10.0, 1.0 and 0.1 µg/ml) significantly inhibits cAMP production (asterisks indicate significant differences; $P \leq 0.0001$, $P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.05$ respectively; one-way ANOVA with a Dunnett post-test) in HEK cells expressing δ-subtype opioid receptors, **D)** which was blocked by the non-selective opioid receptor antagonist naloxone (50 µM) ($P \leq 0.0001$, two-way ANOVA with a Sidak post-test). See also Figure S4. **E)** Venom (50 µg protein/kg i.v.; n=3) causes a single depressor effect on the mean arterial

blood pressure of the anaesthetised rat, but **F)** has no significant effect on heart rate (50 µg protein/kg i.v.; n=3). **G)** Fangblenny venom (2.5 µg protein/ml, n=4) induces a significant decrease (* indicates $P = \leq 0.01$; unpaired *t*-test) in the indirect twitches in the CBCNM preparation over 60 min, but has no effect on the responses to **H)** the exogenous agonists acetylcholine (ACh; 1 mM), carbachol (CCh; 20 µM) and potassium chloride (KCl; 40 mM). All data points displayed represent mean ± SEM.