



ELSEVIER

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Venom on ice: First insights into Antarctic octopus venoms

E.A.B. Undheim^{a,b}, D.N. Georgieva^c, H.H. Thoen^{a,b}, J.A. Norman^b, J. Mork^a, C. Betzel^c, B.G. Fry^{b,*}

^a Norwegian University of Technology and Science, Department of Biology, Trondheim Biological Station, N-7491, Trondheim, Norway

^b Department of Biochemistry & Molecular Biology, Bio21 Institute, University of Melbourne, Melbourne, Victoria 3000, Australia

^c Laboratory of Structural Biology of Infection and Inflammation, Institute of Biochemistry and Molecular Biology, University of Hamburg, Germany

ARTICLE INFO

Article history:

Received 26 November 2009

Received in revised form 12 June 2010

Accepted 16 June 2010

Available online 25 June 2010

Keywords:

Octopoda

Antarctic

Venom

Adaptation

Enzyme

ABSTRACT

The venom of Antarctic octopus remains completely unstudied. Here, a preliminary investigation was conducted into the properties of posterior salivary gland (PSG) extracts from four Antarctica eledonine (Incirrata; Octopodidae) species (*Adelieledone polymorpha*, *Megaleledone setebos*, *Pareledone aequipapillae*, and *Pareledone turqueti*) collected from the coast off George V's Land, Antarctica. Specimens were assayed for alkaline phosphatase (ALP), acetylcholinesterase (AChE), proteolytic, phospholipase A₂ (PLA₂), and haemolytic activities. For comparison, stomach tissue from *Cirroctopus* sp. (Cirrata; Cirroctopodidae) was also assayed for ALP, AChE, proteolytic and haemolytic activities. Dietary and morphological data were collected from the literature to explore the ecological importance of venom, taking an adaptive evolutionary approach.

Of the incirrate species, three showed activities in all assays, while *P. turqueti* did not exhibit any haemolytic activity. There was evidence for cold-adaptation of ALP in all incirrates, while proteolytic activity in all except *P. turqueti*. *Cirroctopus* sp. stomach tissue extract showed ALP, AChE and some proteolytic activity. It was concluded that the AChE activity seen in the PSG extracts was possibly due to a release of household proteins, and not one of the secreted salivary toxins. Although venom undoubtedly plays an important part in prey capture and processing by Antarctica eledonines, no obvious adaptations to differences in diet or morphology were apparent from the enzymatic and haemolytic assays. However, several morphological features including enlarged PSG, small buccal mass, and small beak suggest such adaptations are present. Future studies should be conducted on several levels: Venomic, providing more detailed information on the venom compositions as well as the venom components themselves; ecological, for example application of serological or genetic methods in identifying stomach contents; and behavioural, including observations on capture of different types of prey.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

1.1. Antarctic octopuses

The octopod (Cephalopoda: Octopoda) fauna of the Southern Ocean is both rich and highly endemic, with 12

cirrate (Octopoda: Cirrata) and incirrate (Octopoda: Incirrata) genera currently recognized, of which five genera are endemic (*Adelieledone*, *Bathypurpurata*, *Megaleledone*, *Pareledone*, and *Praealetus* (Collins and Rodhouse, 2006)). However, although several major clades are represented, the Antarctic octopod fauna is dominated by a single eledonine (Incirrata: Octopodidae) lineage which contains 27 of the 36 octopod species currently recognized as part of the Southern Ocean fauna (Allcock et al., 2007; Collins and Rodhouse, 2006). The main radiation is thought to have

* Corresponding author.

E-mail address: bgf@unimelb.edu.au (B.G. Fry).

occurred after the separation of the Antarctic continent and formation of the Antarctic circumpolar current approximately 34 million years ago (Strugnell et al., 2008).

The members of the unique Antarctic octopod clade that are not restricted to the deep sea can be found throughout the Antarctic shelf and continental slope where they have undergone extensive radiation, particularly in the case of *Pareledone*, with each species usually having a fairly limited geographic, and often bathyal, range (Collins and Rodhouse, 2006). While the species radiation has been contributed to the effect of the shelf of isolated island groups combined with glaciation cycles (Allcock et al., 1997, 2001), there are large overlaps in species distributions, with often several species appearing sympatrically in an area (Allcock, 2005). As sympatry is often associated with trophic niche partitioning, this may again lead to a range of adaptations, including venom diversification.

1.2. Venom apparatus and diet

Venoms play a range of adaptive roles in the animal kingdom; killing, paralyzing, immobilizing or pre-digesting prey, as well as defence against predators and deterring competitors (Kordis and Gubensek, 2000). In animals relying on toxins for prey capture and handling, the evolution of the molecular components of venom is often tightly linked to diet and trophic ecology through an evolutionary arms race between predator and prey (e.g. Chetty et al., 2004; Fry et al., 2003; Heatwole and Poran, 1995). Due to the strong selection pressure that results from this arms race, and because toxin genes are members of multigene families (Kordis and Gubensek, 2000), toxins often show very rapid evolutionary rates (e.g. Ohno et al., 2002). The accelerated evolution may be in a diversifying manner, such as conotoxins (Duda and Palumbi, 1999), or convergent, like the fish-specialised venoms of sea kraits (Elapidae; Elapinae) and sea snakes (Elapidae; Hydrophiinae) (Chetty et al., 2004). However, the redundancy in genetic material also means that a niche shift causing a relaxed or loss of selection pressure usually leads to a rapid loss of venom, such as in obligate egg-eating elapid snakes like *Aipysurus eydouxii* (Li et al., 2005b) and *Brachyuropsis* sp. (Fry et al., 2008).

Unlike poisonous animals such as toads, “true” venomous animals (Goyffon, 2002) possess a venom apparatus (glands, secretory tissues, etc.) that includes specialised venom delivery structures. As venom is a widespread feature in the animal kingdom, with every major bilaterian metazoan clade having independently evolved venomous representatives (Fry et al., 2009), the array of structures adapted for envenomation of prey are vast; with examples including teeth (snakes, varanid and helodermatid lizards (Fry et al., 2006)), walking legs (chilopods (Lewis, 1981)), chelate appendages (spiders and pseudoscorpions), caudal segments (scorpions), ovipositors (hymenoptera), antennae (a cerambycid beetle (Berkov et al., 2008)), and radulae (conid gastropods). Along with the rest of the venom apparatus, the evolution of these structures is associated with the degree of dependence upon venom. For example, while the above examples followed the development of toxins, a shift in prey preference

can result in a secondary loss of the venom system e.g. eggs (*A. eydouxii* (Li et al., 2005b), *Dasyplectis* sp. (Fry et al., 2008)) or slugs (*Pareas* sp. (Fry et al. 2008)). However, the venom apparatus is not the only other structure that influences or is influenced by the evolution of venom; the general ability to catch and process the preferred prey by physical force is often an important factor. This may ultimately determine the degree of reliance upon toxins for prey capture, and consequently the strength of the positive selection which drives the recruitment of toxins and evolution of venom in an animal.

1.3. Venom in the Octopoda

One group that uses venom in the capture of otherwise tricky or potentially dangerous prey is members of the Octopodidae, which are known to prey on a range of taxa, including molluscs, crustaceans, fish, and even birds (Grubert et al., 1999; Sazima and Bastos-de-Almeida, 2006). Although small prey is often handled without the use of venom, many octopods switch to the use of venom once the use of physical force becomes inefficient (Fiorito and Gherardi, 1999; Grisley et al., 1999). In large, shelled prey, for example, small holes are often drilled through which the octopus injects its toxic saliva, which then acts by paralyzing and/or killing the prey as well as aiding in the detachment of tissue from the exoskeleton (Grisley and Boyle, 1987; Nixon, 1984; Pilson and Taylor, 1961).

The production of toxic saliva in octopod posterior salivary glands (PSG) was first recognized at the end of the 1800s (Ghiretti, 1960). Despite findings as early as 1906 that the toxin was a protein particularly potent against crustaceans (Songdahl and Shapiro, 1974), it was long thought that the paralysis and death observed in envenomated prey was due to the actions of various amines isolated from octopod PSG, such as tyramine, histamine, acetylcholine, octopamine and serotonin (Erspamer and Asero, 1953; Ghiretti, 1960; Henze, 1913). However, the findings that these amines at realistic concentrations only caused the initial symptoms of “overexcitability” in envenomated crabs, and were unable to reproduce the irreversible paralyzation achieved by injection of crude saliva, suggested a more complex venom composition (Ghiretti, 1959). This resulted in the description of Cephalotoxin (Ctx), a protein mixture consisting of four proteins, including at least one glycoprotein, originally extracted from the PSG of the cuttlefish *Sepia officinalis*, and later *Octopus vulgaris* (Ghiretti, 1959, 1960). Ctx was reported to have a number of activities, including inhibition of respiration in crabs, inhibition of blood coagulation in both crabs and humans, and paralyzation of crabs and cockroaches (Ghiretti, 1960).

Since then, several proteinaceous toxins have been isolated and their activities described from the salivary glands from a range of octopodid species, for example *Eledone cirrhosa* (e.g. Grisley, 1993), *Hapalochlaena maculosa* (e.g. Sheumack et al., 1978), *Octopus dofleini* (Songdahl and Shapiro, 1974), and *O. vulgaris* (e.g. Kanda et al., 2003). Being responsible for the serious symptoms (even human fatalities) associated with bites from members of *Hapalochlaena* (e.g. Cavazzoni et al., 2008), and

proving useful in areas like clinical research (Lewis and Garcia, 2003), much of cephalopod toxicological research has been directed towards tetrodotoxin (TTX) and TTX-like compounds (Fry et al., 2009). However, studies have shown that like in many other marine organisms, *Hapalochlaena* TTX is produced by endosymbiotic bacteria and distributed not only in the salivary glands, but also all other parts of the body (Yotsu-Yamashita et al., 2007).

Relatively little is known about the presumably endogenous salivary toxins found in octopods. It seems, however, that various proteases and neurotoxins are important venom constituents. In a recent study, Fry et al. (2009) found large numbers of peptidase S1 transcripts in both *H. maculosa* and *Octopus kaurna*, as well as evidence for a basal radiation of these transcripts in the Cephalopoda. Moreover, the molecular diversity in the functional residues of the encoded proteins coheres to the pattern seen in multigene toxin families that have undergone adaptive radiation through positive selection, such as conotoxins (Duda and Palumbi, 1999; Fry et al., 2009). This basal radiation and functional diversification is consistent with observations on protease activities in octopodid species belonging to phylogenetically relatively distant subfamilies (Strugnell et al., 2005), such as the eledonine *E. cirrhosa* (Grisley, 1993) and octopodine *O. vulgaris* (Morishita, 1974), where ten and eight, respectively, caseinolytic proteases were identified.

Like proteases, neuro/myotoxins seem to be a common feature of most octopodid venoms. Small neuropeptides with potent hypotensive properties have been isolated from PSG extracts from species belonging to both Eledoninae and Octopodinae; eledoisin from *Eledone aldrovandi* and *Eledone moschata* (Erspamer and Anastasi, 1962), Octopus tachykinins (OctTK) from *O. vulgaris* (Kanda et al., 2003), and a peptide-transcript in *O. kaurna* homologous to that coding for OctTK (Fry et al., 2009). Similarly, non-TTX-like neuro/myotoxic proteins have been found in PSG extracts from *O. dofleini* (Songdahl and Shapiro, 1974), *O. vulgaris* (Ctx), and *E. cirrhosa* (McDonald and Cottrell, 1972).

In the Octopoda, the venom apparatus terminates in the toothed salivary papilla, which is connected to the paired PSG through ducts running adjacent to the oesophagus (Grisley et al., 1996). As well as probably functioning as a reservoir, the salivary papilla is used to inject saliva into prey, often with great force, through either puncture wounds caused by the beak or holes drilled mainly by the physical actions of the radula (Runham et al., 1997). This results in immobilization of prey, such as by hypotension and paralysis followed by death in crustaceans (Ghiretti, 1960), but seemingly only temporary paralysis or hypotension in molluscs (Pilson and Taylor, 1961). In addition to the enlarged and well developed PSG found in species relying on the use of venom (Gibbs and Greenaway, 1978), taxa that bore holes also have other evident morphological adaptations, including a concentration of sensory nerve endings near the tip of the radula, a particularly well developed sub-radular organ, and an extra supra-radular organ which functions are to remove old radular teeth (Messenger and Young, 1999). However, it is not known how the venom composition itself is influenced by the

dietary preferences of octopods, nor whether octopods with less robust morphology and physical strength “compensate” in terms of toxicity of their venom.

1.4. Venom enzymes and temperature

Most enzymatic venom components for which the effect of temperature on activity has been described appear to be mesophilic enzymes: Thermal optima range between 25 °C (Hernandez-Oliveira et al., 2005) and 65 °C (Pandya and Budzynski, 1984) but are usually within 37–55 °C. Optima do not seem to differ much between enzyme families except for snake phosphodiesterases which with thermal optima typically about 60 °C are in the higher end of the scale (Valério et al., 2002). In addition to different types of enzymes having similar thermal activity properties within each species (e.g. Liu et al., 2006; Liu et al., 2008; Sun et al., 2009), the window of optimal temperatures also seems to be consistent across a wide range of venomous taxa, including snakes (e.g. Costa et al., 2010; Farid et al., 1989), fish (Magalhães et al., 2008; Poh et al., 1992), spiders (Devaraja et al., 2008; Rodrigues et al., 2006), scorpions (Feng et al., 2008; Morey et al., 2006), insects (de Jong et al., 1982; Zhu et al., 2008), and jellyfish (Li et al., 2005a). As with other mesophilic enzymes, the activity rates of these enzymatic toxins tend to drop rapidly with a decrease in temperature, often with 10–20% activity at 20 °C and virtually no activity below 4 °C. Unlike previously described enzymatic toxin, however, the enzymes in the venom of Antarctic octopods are subjected to a set of environmental conditions very different from the venoms of tropical or terrestrial temperate organisms which need to function under warm conditions (such as endothermal prey).

1.5. Aims

During participation in the Australian Antarctic Division's third International Polar Year research cruise, “Voyage 3”, onboard the R/V “Aurora Australis” to Commonwealth Bay, Eastern Antarctica, a large number of octopods were collected. As well as being a unique opportunity for an investigation of the properties of venoms used under sub-zero conditions, where most enzymes largely lose their function, it also provided a chance to compare largely sympatric species of various relatedness in terms of toxic activity, morphology and diet.

In the present study, we provide the first insights into the properties and importance of venom in Antarctic Incirrata, shedding some light on how octopod venom composition may be influenced by diet and morphology. The underlying hypothesis was that venom is an important part of Antarctic octopod trophic ecology, and that there is a strong link between venom composition, morphology and diet. Hence, it might be expected that the size of prey and difficulty in capturing is reflected in the morphology and venom of a species, with a lack of strength in one being compensated for in the other. In addition, due to their radiation during and after the formation of the Antarctic circumpolar current as well as considering the adaptive nature of most toxin families, Antarctic octopodid venom is

likely to exhibit adaptations to functioning under sub-zero conditions.

2. Material and methods

2.1. Sample collection

During the Australian Antarctic Division's third International Polar Year research cruise "Voyage 3" onboard the R/V "Aurora Australis", 203 specimens of octopods were collected using benthic beam trawls and box corer (one individual). These were obtained from 52 of a total of 78 stations sampled off the coast of Eastern Antarctica, during the six week duration of the cruise.

All specimens were photographed, locality and depth recorded, and a tissue sample taken from the tip of a non-hectocotylized arm. The tissue samples were put in liquid nitrogen for storage, while the specimens were deposited with the Collaborative East Antarctic Marine Census (CEAMARC) collection, each specimen and its tissue sample given a unique CEAMARC reference number. Posterior salivary glands (PSG) were collected from specimens designated by the shift leader (CEAMARC) and stored in liquid nitrogen. Removal of the PSG was carried out by a dorsal incision through the mantle immediately posterior to the eyes (Fig. 1), revealing the paired glands on either side of the oesophagus.

Identification of specimens identified 15 species groups, nine of which could be positively assigned to named species. Species to be included in the venom analyses were selected so to include varying degrees of relatedness, as well as based on their ecological and morphological traits. Specimens included in the study are listed in Table 1 along with their designated CEAMARC numbers and catch data, and their phylogenetic relationships as described by Undheim et al. (2010) are illustrated in Fig. 2.

2.2. Venom analyses

As the term implies, enzymatic assays detect and estimate enzymatic activities by providing a substrate (natural form or

analogue) and measuring the concentration of the products over time. By testing for a variety of enzymatic and other activity assays, both prey immobilizing and pre-digesting effects can be predicted. Because of the precious nature of the material and to enable further, more detailed work, only one specimen could be included from *Megaleledone setebos* and *Pareledone turqueti*, and three specimens from *Adelieledone polymorpha* and *Pareledone aequipapillae*.

2.2.1. Protein extraction

A "Total Protein Extraction Kit" (Millipore® Cat. #2140) was used for the extraction of proteins from posterior salivary glands (PSG), pooling conspecific extracts. All extraction was done in a cold room keeping 4 °C. Buffer containing protease inhibitors was created and kept on ice. PSG tissue was weighed, and approximately half of the total 2.5 mg/μL buffer added before homogenizing the tissue for 20 s using a manual tissue homogenizer, put on ice for 15 s, and repeated three times. The homogenizer and edge of the homogenizer-tube were then rinsed off using the remaining buffer before allowed to rest on ice for 20 min, but manually shaken for 5 s every 2 min. The extract was then transferred to an Eppendorf tube and centrifuged at 4 °C for 30 min at 11 000 rcf (relative centrifugation force), the resulting supernatant transferred to a 0.1 μm filtered spin column. The pellet was resuspended in 200 μL buffer and set on ice for 20 min, manually shaken every 2 min, before centrifugation at 4 °C for 30 min at 11 000 rcf. The second supernatant was then added to the first, and concentrated in the spin column by centrifugation for approximately at 4 °C for 20 min at 7000 rcf, checking the total volume every 5–10 min. The total protein concentration of all extracts used in assays were estimated using a Bradford (1976) Assay.

2.2.2. Alkaline phosphatase Assay

ALP is an enzyme with relatively high pH optimum that is widely distributed, both in terms of taxa and tissues (McComb et al., 1979), and has been found in the venoms of spiders (Heitz and Norment, 1974; Rodrigues et al., 2006) and snakes (Sulkowski et al., 1963; Tu and Chua, 1966). As it is not particularly substrate specific, ALP is known to hydrolyze a wide variety of esters and anhydrite phosphoric acids to release phosphate (Guimarães et al., 2001). By playing a part in the endogenous release of purines during envenomation, ALP is thought to contribute to the immobilization of prey through hypotension (Aird, 2002). To test for the presence of ALP, a slightly modified assay from Sulkowski et al. (1963) was used for detecting and measuring ALP activity.

For each extract, an assay and a control was prepared by adding the following to two Eppendorf tubes: 100 μL 0.01 M glycine–NaOH buffer pH 9, 120 μL 0.01 M *p*-nitrophenylphosphate (PNPP), 30 μL 1.00 M MgCl₂, 40 μL H₂O. 10 μL of extract and 10 μL of H₂O, as control, was then added to their respectively labelled tubes and immediately placed on a thermo-shaker preheated to 37 °C or on ice. After exactly 30 min, 300 μL 0.05 M NaOH was added in order to terminate the reaction.

The two samples were then measured for absorption at 400 nm (A₄₀₀) using a NanoDrop® ND-1000

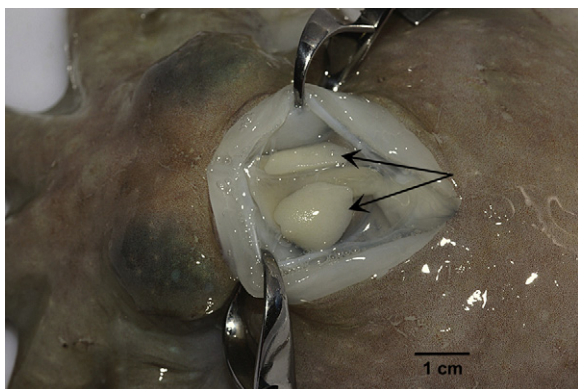


Fig. 1. Dorsal view of the mantle of *Pareledone turqueti* showing the location of the paired posterior salivary glands, indicated by arrows, on either side of the oesophagus, immediately posterior of the eyes. Notice the three layers through which the incision has been made.

Table 1

CEAMARC number and catch data for specimens included in the venom analyses. Total length is the total length of the specimen from tip of mantle to end of longest arm, trawl depths are at start and stop, while coordinates indicate the locations of the set CEAMARC sampling stations. The presence of a hectocotylus (Hect.) shows specimen is a mature male; an absence may indicate both immature individual and mature female.

Species	CEAMARC Voucher number	Hect.	Total length (mm)	Trawl depth (m)	Latitude	Longitude
<i>P. turqueti</i>	CEAMARC V3 2716	Yes	145	791–684	66°23.940'S	140°31.780'E
<i>P. aequipapillae</i>	CEAMARC V3 1651	No	140	404–414	66°35.930'S	145°23.365'E
	CEAMARC V3 1656	No	125	404–414	66°35.930'S	145°23.365'E
	CEAMARC V3 1929	Yes	140	566–561	66°20.417'S	143°39.900'E
<i>M. setebos</i>	CEAMARC V3 2801	No	980	444–331	66°19.856'S	140°39.831'E
<i>A. polymorpha</i>	CEAMARC V3 497	?	150	390–294	66°20.020'S	142°43.683'E
	CEAMARC V3 740	?	151	384–385	66°33.742'S	142°22.689'E
	CEAMARC V3 3603	No	100	Ca. 748	65°41.489'S	140°31.673'E
<i>Cirroctopus</i> sp.	CEAMARC V3 3120	No	620	414–402	66°10.021'S	139°41.057'E

Spectrophotometer with software NanoDrop3.1.0, noting the difference in A_{400} between the control and extract. Specific activity was calculated as the amount of enzyme which liberates 1 μmol of *p*-nitrophenol per minute per mg protein in the extract. The activity was measured in triplicate and averaged values were taken.

2.2.3. Acetylcholinesterase assay

In its ancestral state, AChE most commonly found as an extrinsic membrane protein that terminates the acetylcholine-receptor mediated ion gating in the post-synaptic neuron by hydrolyzing acetylcholine (ACh) (Quinn, 1987). It is extremely efficient and has one of the highest turnover rates registered for any enzyme, hydrolyzing ACh faster than its diffusion rate (Quinn, 1987). Although found in high levels in many elapid snake venoms, the toxic role of AChE is uncertain (Cousin et al., 1996). It is thought, however, that it results in less muscular control by rapidly hydrolyzing ACh (Fry, 2005) and, like ALP, plays a role in immobilizing prey due to hypotension, although in an indirect manner (Aird, 2002).

Alkaline hydroxylamine was prepared by adding equal parts of 1.7 M NaOH and 1 M hydroxylamine-HCl. Six 1.5 ml Eppendorf tubes, one for each sample and one for control, were labelled and added 10 μL sample and 10 μL H₂O (control). 2 μL 1 M MgCl₂ was then added before incubating the tubes for 3 min at 37 °C. To initiate the reaction, 20 μL 0.0064 M acetylcholine chloride was added followed by incubation at 37 °C on a thermo-shaker.

After exactly 10 min 80 μL of the freshly prepared alkaline hydroxylamine was added to terminate the reactions. 40 μL 1:6 grad HCl and 40 μL 0.185 M ferric chloride were then added, carefully mixing with the pipette. The resulting rusty-purple colour was then measured for absorption at 540 nm using a NanoDrop spectrophotometer.

2.2.4. Proteolytic assay

Although not a natural substrate for venom proteases, casein gives an indication of general proteolytic activity and is commonly used in the detection of proteases in venoms from a range of taxa (e.g. Gusmani et al., 1997; Matsui et al., 2000), including octopods (Grisley, 1993). Because of the addition of protease inhibitors during the standard protein extraction protocol (see 2.2.1) separate extracts had to be prepared before running this assay. Same extraction

procedure was followed, except no protease inhibitors were added to the extraction buffer. This meant that the extracts needed to be prepared immediately prior to running the proteolytic assay, alternatively frozen at –80 °C and thawed as the assay was set up.

A modified assay based on that by Johnson et al. (1969) was used for detecting and measuring the presence of proteolytic activity. The substrate of the reaction, casein, was prepared by adding 1.2 g casein to 70–80 mL of the phosphate buffer, letting it sit for 10–15 min, placing the beaker containing the casein mixture in a boiling water bath, and stirring at 300 rpm for 20–30 min until all casein was dissolved. The casein solution was then allowed to cool down to room temperature before adding phosphate buffer until the total volume of the casein solution reached 100 mL. Because of the low solubility of casein, the solution was filtered before use in order to remove particles.

Phosphate buffer pH 7.4 was prepared from 0.1 M NaH₂PO₄·2H₂O and 0.15 M NaCl by adding 6.9 g NaH₂PO₄ and 4.4 g NaCl to 500 ml H₂O. The pH of the phosphate buffer was regulated by adding NaOH while monitoring the pH until it reached 7.4. Assays were initiated by adding 20 μL extract to 50 μL casein solution, placed on a shaker for 10 min. To terminate the proteolytic reaction casein was precipitated by adding 50 μL HClO₄, made from 39 mL HClO₄ and 500 mL H₂O, and placing the solution on a shaker for 20 min. Due to the differences in absorption between the extractions, each sample needed its own control, where order of adding casein and HClO₄ was reversed. This meant extract enzymes were not given any time to digest the casein before it was precipitated. The assay was carried out at two temperatures, 0 °C and 37 °C. One (37 °C) being the standard protocol temperature, while the other (0 °C) being closer to the conditions of the species' natural environment.

Each tube was then centrifuged at maximum speed (16 100 rcf) at 4 °C for 30 min. The resulting supernatants were measured for absorbance at 280 nm using the NanoDrop, noting the difference in A_{280} between control and extract. Specific activity was calculated as the number of Committee of Thrombolytic Agents (CTA) units per milligram total protein, where one CTA unit liberates 0.1 micro equivalents of tyrosine from cow casein in 1 min at 37.5 °C. The activity was measured in triplicate and averaged values were taken.

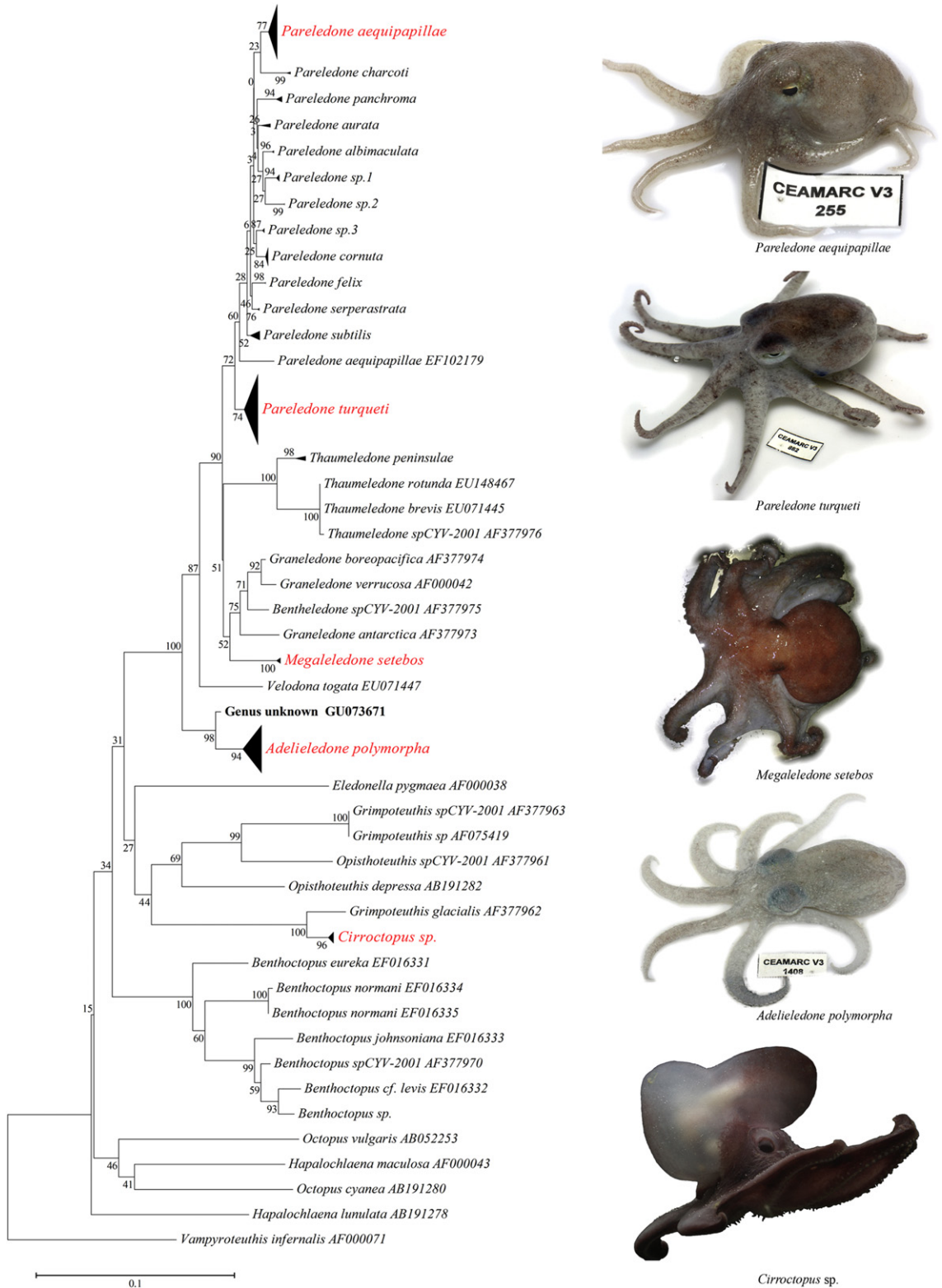


Fig. 2. K2P distance based NJ phylogenetic tree analysis of sample COI sequences onto a template of COI sequences obtained from GenBank (Undheim et al., 2010). Names of species obtained are in bold, while species selected for further study are underlined. Scale represents a genetic distance of 0.1 units, which corresponds to an average of 0.1 nucleotide substitutions per nucleotide.

2.2.5. Secreted Phospholipase A₂ assay (sPLA₂)

Venom sPLA₂ is found in a whole range of taxa, including cone snails (McIntosh et al., 1995), bees (Tracy et al., 1980), and snakes (Fry and Wuster, 2004). Although the downstream effects are diverse, three main mechanisms inducing pharmacological effects have been proposed (Rosenberg, 1990): i) Hydrolysis of membrane phospholipids to directly disrupt functions such as selective membrane permeability of cells. ii) Hydrolysis of membrane phospholipids to produce pharmacologically active products, such as arachidonic acid, which then mediate PLA₂ actions like inflammation and tissue destruction (Fry et al., 2005). iii) Direct membrane disruption, independent of phospholipid hydrolytic activity, leading to for example haemolytic activity.

A Cayman Chemical secretory Phospholipase A₂ (sPLA₂) Assay Kit (<http://www.caymanchem.com/app/template/Product.vm/catalog/765001/a/z>) was used to measure the specific activity of the sPLA₂s in the crude venom extracts. Conducting the assay at two temperatures, one being 0 °C, was attempted but failed due to the requirements for real-time measurement of absorbance at 405 nm (A₄₀₅). The negative control was used to establish the baseline of the activity to which the slopes, and hence activities, of the remaining reactions were estimated. The activity was measured in triplicate and averaged values were taken.

2.2.6. Haemolytic assay

Haemolysis is the eruption of red blood cells and the subsequent release of cell contents, such as haemoglobin, into the plasma. With several toxin types (both enzymatic and non-enzymatic) exhibiting some form of haemolytic activity (Chen et al., 1997), it is a common effect found in venoms from a vast array of taxa, ranging from snakes (Fry, 2005) to cnidarians (Gusmani et al., 1997), and to octopods (Key et al., 2002). Although haemolysis may have several systemic effects, such as disseminated intravascular coagulation and renal failure (Vetter, 2008), its detection in assays can also be an indicator of general cytolytic activity, which may again act as a spreading factor for the remaining toxins.

To test for haemolytic activity in the extracts, a modified assay adopted from Key et al. (2002) was used. Phosphate buffered saline (PBS), pH7.3, was prepared by adding 8 g NaCl, 0.2 g KCl, 0.2 g KH₂PO₄, 1.725 g Na₂HPO₄·7H₂O, 1L distilled H₂O, and adjusting the pH to 7.3. Mammalian blood was then centrifuged for 5 min at 12 000 rpm, the supernatant removed, and the red blood cells (RBC) resuspended in an amount of PBS equal to that of the supernatant removed. The RBC were centrifuged and “washed” with PBS three times in order to reduce the amount of non-erythrocyte matter present.

An appropriate RBC concentration was determined by setting up a concentration series of 50%, 25%, 10%, 5%, 2%, and 1.5% of original RBC density using PBS, and inducing 100% haemolysis by re-suspending the RBC in distilled water. The mixtures were then centrifuged for 5 min at 12 000 rpm, and the supernatant measured for absorption at 540 nm (A₅₄₀). Due to 100% absorption at higher concentrations, the 1.5% RBC solution was decided to be the most appropriate. A haemolytic percentage curve was set up by diluting the 100% haemolytic mixture to 50%, 25% and 12.5% and measuring A₅₄₀, and then solving the linear

best-fit line equation for X in order to estimate % haemolysis from the observed A₅₄₀: $x = (y + 0.21)/0.013$.

To minimize the error rate from inaccurate substrate concentration, the same solution used for calibrating the absorbance to % haemolysis curve was used for assaying the extracts. 50 µL extract was added to 500 µL 1.5% RBC in 1 mL Eppendorf tubes. For blank, 50 µL PBS was added instead of extract. The tubes were then incubated at room temperature (approx. 20 °C) on a rotator for 2 h, before centrifugation at 12 000 rpm for 5 min, and measuring A₅₄₀ of the supernatant. The assay could only be performed once due to the lack of material, preventing calculation of the variation associated with the assay protocol. In addition, the lack of resources and time prevented estimation of expected standard deviation of the assay results, which could not be found in the literature.

2.2.7. SDS-PAGE

To visualize the contents of the extracts, samples were run through an SDS-PAGE. A 14% poly-acrylamide gel was used to get higher band-resolution in the area containing the smaller proteins. This approach, however, also resulted in lower resolution among larger-sized proteins. Due to previous attempts at running SDS-PAGE using lower protein concentrations and silver staining, it was decided not to spend more extract on SDS-PAGE and instead save as much as possible for further, more informative separation methods. The extracts were prepared by adding 10 µL SDS-buffer to equal parts extract and set for incubation for 5 min at 96 °C on a thermo-shaker, added to a pre-made gel, focused for 30 min at 120 V in a 4% pre-gel, and run for 90 min at 180 V. The resulting gel was stained in CBB on a shaker at room temperature before rinsed in a buffer containing 40% EtOH, 10% Acetic Acid, and H₂O.

2.3. Morphology

Information on characters considered relevant to prey capture and use of venom was collected from the literature. Two characters, the radular apparatus and the salivary papilla, could not be included as no observations were made during specimen collection or was found in the literature. The following morphological features were therefore included in the interspecific comparison: PSG shape and size, beak shape, size of the buccal mass in relation to mantle length, sucker count in relation to arm length, and any notes on the muscularity or robustness of the body. All traits except PSG shape and size were used as indicators of physical ability to subdue prey; while properties of the beak and buccal mass reveal the ability to crush and bite chunks out of prey, the number of suckers per arm length often indicates the sucker size and hence ability to handle small prey.

2.4. Diet

Dietary data had to be taken from the literature as stomach contents could not be recorded due to the partitioning of samples and data between different research groups participating on the cruise. However, very little is known about the diet of Antarctic octopods; cephalopod stomach contents are in general often difficult to identify

due to the maceration of prey prior to ingestion, and the lack of knowledge about the benthic shelf fauna of the Antarctica makes a detailed description of any visually recognizable items virtually impossible (Collins and Rodhouse, 2006). Nevertheless, three studies have been published which give general identifications of stomach contents from the cirrate (Vecchione et al., 1998) and incirrate (Daly, 1996; Piatkowski et al., 2003) species, or closely related congeners, included in the venom analysis. Although these records are from the western Antarctic region, they provide a general comparative picture of diets of octopods inhabiting areas with an unusual benthic faunal assemblage.

3. Results

3.1. Activity assays

The results obtained from the ALP assay are summarized in Table 2. It is evident that all extractions have some form of alkaline phosphatase present, with positive results at both 37 °C and 0 °C. Three of the four species also showed higher activities at 0 °C than 37 °C. *P. aequipapillae*, however, exhibited 60% higher activity rate at 37 °C than at the more ecologically realistic temperature of 0 °C.

In contrast, little or no acetylcholinesterase activity was found in assays conducted at 37 °C, except *P. aequipapillae*, which showed a ten-fold increase in activity at 37 °C compared to 0 °C. All species had low activities at 0 °C, but the results were very inconsistent, including highly variable control values, and the results were consequently excluded from the analyses.

The results obtained from the proteolytic assays are summarized in Table 3. All species except *P. turqueti* showed higher or approximately equal activities at 0 °C compared to 37 °C, with *M. setebos* having the highest rate of activity at 0 °C. *P. turqueti* showed a specific activity similar to that of *M. setebos* and *P. aequipapillae* at 37 °C, but near zero activity at 0 °C.

The sPLA₂ assay was only conducted at 29 °C, at which all extracts showed some PLA₂ activity, the highest rate being that of *P. turqueti* which had a rate more than twice than the congeneric *P. aequipapillae*. sPLA₂ assay results are summarized in Table 4.

Except for *P. turqueti*, all PSG extracts had weak haemolytic activity. Results from the haemolytic assay are summarized in Table 5.

3.2. Cirroctopus stomach tissue

The protein extract from the stomach tissue of *Cirroctopus* sp. was assayed for some, but not all of the

Table 2

Alkaline phosphatase (ALP) activity at 0 °C and 37 °C of Antarctic octopus venoms. Specific ALP activity is defined as units per mg total protein in extract. 1 Unit ALP activity represents the amount of enzyme which liberates 1 μmole of *p*-nitrophenol per minute.

Species	Specific activity at 0 °C (U/mg)	Specific activity at 37 °C (U/mg)
<i>A. polymorpha</i>	69.32 ± 3.1	61.20 ± 3.0
<i>M. setebos</i>	86.78 ± 4.3	57.60 ± 2.9
<i>P. aequipapillae</i>	116.22 ± 4.0	166.13 ± 3.8
<i>P. turqueti</i>	99.08 ± 3.3	76.95 ± 3.2

Table 3

Proteolytic activity at 0 °C and 37 °C of Antarctic octopus venoms. Specific proteolytic activity is defined as proteolytic units per mg total protein in extract. One proteolytic unit represents one CTA unit which liberates 0.1 micro equivalents of tyrosine from cow casein in 1 min at 37.5 °C.

Species	Specific activity at 0 °C (U/mg)	Specific activity at 37 °C (U/mg)
<i>A. polymorpha</i>	1.32 ± 0.06	0.56 ± 0.03
<i>M. setebos</i>	5.05 ± 0.15	2.60 ± 0.09
<i>P. turqueti</i>	0.22 ± 0.01	2.45 ± 0.09
<i>P. aequipapillae</i>	3.25 ± 0.1	2.9 ± 0.1

enzymatic groups, due to the low protein content of the extract. The results from the assays conducted are summarized in Table 6, which shows that ALP and proteolytic activity is present, albeit low in the latter.

3.3. SDS-PAGE

The result from the SDS-PAGE is shown in Fig. 3. In addition to the abundance of peptides too small for separation, the gel showed a strong band at approximately 12–13 kDa for *P. turqueti* and *P. aequipapillae*, whereas weak bands were present in this region for both *A. polymorpha* and *M. setebos*. *M. setebos* also showed definite bands at 14 kDa and 18 kDa. Considering the results from the activity assays, these bands correspond to the molecular weight range of sPLA₂, which commonly is between 13.5 and 16 kDa.

A couple of weak bands were present in the region 25–30 kDa for *A. polymorpha*, *P. turqueti*, *P. aequipapillae*, as well as the stomach tissue extract from *Cirroctopus* sp. All PSG extracts also had very weak bands at about 35 kDa.

Strong, broad bands along with 1–3 weaker bands indicate the presence of several proteins around 45 kDa in *A. polymorpha*, *P. turqueti*, and *P. aequipapillae*. The stomach tissue extract of *Cirroctopus* sp. also contained proteins in this region, though somewhat larger than 45 kDa. Although no ALP activities have been reported from octopod venom, the molecular weights described for this phosphatase family have generally been higher than 30 kDa (Rodrigues et al., 2006). Considering the activities present in both the PSG and stomach tissue extracts, the bands at about 45 kDa may represent ALP enzymes. The lack of strong bands in the *M. setebos* extract above 18 kDa but positive results in the proteolytic and ALP assays suggest either that some of the protein content of the extract may have degraded while running the gel, or that activities of these enzymes are very high.

Table 4

Phospholipase A₂ (PLA₂) activity of Antarctic octopus venoms. Specific sPLA₂ activity is expressed as sPLA₂ enzyme activity units per mg total protein in extract, where one unit hydrolyzes 1 μmol of diheptanoyl Thio-PC per minute at 25 °C.

Species	Specific PLA ₂ activity (mg protein) ⁻¹ (U/mg)
<i>A. polymorpha</i>	0.44 ± 0.02
<i>M. setebos</i>	0.77 ± 0.03
<i>P. turqueti</i>	1.21 ± 0.05
<i>P. aequipapillae</i>	0.66 ± 0.03

Table 5

Relative and specific relative haemolytic activity for each species. Values denote the percentage haemolysis by extracts compared to 100% haemolysis induced by distilled water in a 1.5% dilution of mammalian red blood cells. Specific relative activity is defined as the relative activity per mg total protein in each extract. Variation due to measurements of absorbance gave a standard deviation of 4.3%.

Species	A ₅₄₀	% Haemolysis	% Haemolysis per mg protein
<i>A. polymorpha</i>	0.023	3.38	0.615
<i>M. setebos</i>	0.057	6.00	2.00
<i>P. turqueti</i>	0	0	0
<i>P. aequipapillae</i>	0.072	7.15	1.43
PBS only	0	0	N/A

3.4. Morphology

The size and shape of the PSG and beak of *A. polymorpha* is very different in comparison to the other incirrates. *A. polymorpha* also differs by having smaller buccal mass and suckers, but resembles *M. setebos* in arm length and shape although the webbing is not as deep. The main difference between *M. setebos* and the two species of *Pareledone* is the large size of *M. setebos* and its thick but very loose and gelatinous integument. However, the stout build and deep webbing combined with its large size (largest specimen TL 980 mm) makes *M. setebos* a massive octopus difficult to compare with its smaller eledonine relatives. The two members of *Pareledone* only really differ in the length of their arms and texture of their integument, with *P. turqueti* having longer, seemingly thicker arms and smooth skin. In comparison to the papillated skin of *P. aequipapillae*, *P. turqueti* appears more gelatinous, although not as much as *A. polymorpha*.

Compared to its incirrate counterparts, the cirrate *Cirroctopus* sp. has a very different build. In addition to a very gelatinous body (perhaps particularly in large specimens) and the use of “wings” as the primary form of locomotion, *Cirroctopus* lack radulae, have no PSG, have two rows of cirri lining each side of the suckers, and possibly have only weakly chitinised beaks (Kubodera and Okutani, 1986; but see Vecchione et al., 1998).

Hence, as apparent from the results summarized in Tables 7 and 8, there seems to be three main morphological groups. One, composed of *P. aequipapillae*, *P. turqueti*, and *M. setebos*, generally fit into the typical morphology of

Table 6

Results from enzymatic assays on crude protein extract from the stomach tissue of *Cirroctopus* sp. Units are as follows: 1 Unit ALP activity represents the amount of enzyme which liberates 1 μmole of *p*-nitrophenol per minute; 1 Proteolytic unit represents one CTA unit which liberates 0.1 micro equivalents of tyrosine from cow casein in 1 min at 37.5 °C; Haemolytic values denote the percentage haemolysis by extracts compared to 100% haemolysis induced by distilled water in a 1.5% dilution of mammalian red blood cells. Specific activity is defined as units per mg total protein in each extract.

Enzymatic Assay	Specific/Relative activity
Alkaline phosphatase (ALP)	76.09 ± 3.3 U/mg
Acetylcholinesterase	Weak
Proteolytic assay	0.38 ± 0.02 U/mg
Haemolytic assay	0%

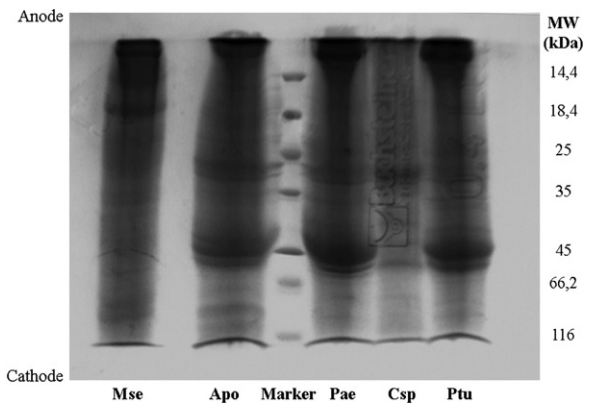


Fig. 3. SDS-PAGE of all extracts with the following abbreviations; Mse = *M. setebos*, Apo = *A. polymorpha*, Pae = *P. aequipapillae*, Cma = *Cirroctopus* sp., Ptu = *P. turqueti*.

eledonine octopods, with a typical beak shape, “normally” sized PSG, and slightly variable sizes and appearances of buccal mass, arms, suckers, and integument. *A. polymorpha*, however, has a very atypical shape and size of both the beak and PSG, and has a fairly small buccal mass. The third group, which consists of *Cirroctopus* sp., is again completely different from the remaining species. This is not surprising, however, as it is a member of Incirrata, which diverged from the Cirrata approximately 215 million years ago (Strugnell et al., 2008).

3.5. Diet

Most notable in the dietary components registered for Antarctic incirrates is the lack of molluscs; no molluscan remains were identified from the stomach contents of any of the species apart from *P. turqueti*. Molluscs are, alongside crabs, often one of the preferred types of prey in octopods (Ambrose, 1984; Ambrose and Nelson, 1983; Grubert et al., 1999), and the absence of brachyurans from the Antarctic continental shelf fauna (Clarke, 2008) therefore suggests that molluscs would be an important component of Antarctic octopods’ diets. However, because the hard exoskeleton of large shelled prey is rarely ingested by octopods, molluscan, and particularly bivalve, remains are probably particularly difficult to identify without employing molecular methods. Piatkowski et al. (2003), for example, found no gastropod nor bivalve remains, but were also unable to identify 44% of *P. turqueti* stomach contents. This large proportion of the diet is not unlikely to include shelled molluscs, which was identified as prey in an earlier study by Daly (1996), and are known to be a quite diverse and occasionally substantial part of the Antarctic shelf fauna (Allcock et al., 2003c; Clarke, 2008).

P. turqueti seems to exhibit an opportunistic and generalist diet more typical of octopods (Ambrose, 1984; Ambrose and Nelson, 1983; Grubert et al., 1999). Although, as mentioned, a large proportion could not be determined, identified prey items include amphipods, polychaetes, fish, octopods, and crustaceans in addition to gastropods and bivalves. This cannot be said for all

Table 7

A comparison of morphological features considered important in prey capture, and for which observations were made or found in the literature. Abbreviations: TL = Total body length.

Species	Size (Max TL) (mm)	Posterior salivary gland	Beak shape and size	Buccal mass size	Relative sucker size	Arm length	Notes
<i>P. aequipapillae</i>	210	PSG length same as buccal mass ^a ; Max PSG length approx. 7% TL; Triangular shape	Normal octopod shape, medium size ^a	As <i>P. turqueti</i> ?	As <i>P. turqueti</i> ^{a,c}	Medium (approx. 60% TL) and tapering ^a	Body more robust than <i>P. turqueti</i> ^f ; Arms shorter and not as thick.
<i>P. turqueti</i>	312	PSG length same as buccal mass ^b ; Max PSG length approx. 7% TL; Triangular shape	Normal octopod shape, medium size ^b	Significantly larger than <i>A. polymorpha</i> ^e	Significantly larger than <i>A. polymorpha</i> ^e	Long (approx. 65% TL) and tapering ^f	Slightly gelatinous ^f , but more robust than <i>A. polymorpha</i> ^e
<i>M. setebos</i>	980	Max PSG length approx. 7% TL; Triangular shape	Normal octopod shape, large size ^d	Large, but relative size similar to <i>P. turqueti</i> ?	Slightly larger than <i>Pareledone</i> ^c	Long (approx. 67% TL) and stout ^f	Integument thick ^d , loose and gelatinous ^f ; Deep webbing ^{b,d}
<i>A. polymorpha</i>	200	PSG length almost twice that of buccal mass ^b ; Max PSG length approx. 10% TL; Square shape ^b	Sharply pointed rostrum, small size ^b	Significantly smaller than <i>P. turqueti</i> ^e	Small ^e	Moderately long (approx. 62% TL) and stout ^b	Less robust than members of <i>Pareledone</i> ^e
<i>Cirroctopus</i> sp.	620	Absent ^g (within the buccal mass? ^h)	Normal octopod shape ^h , weakly chitinised ⁱ	Medium, but lacks radula ^g	Medium to small ^g ; Row of cirri on both sides of suckers	Medium ^g (approx. 55% TL)	Very gelatinous; "Wings" primary mode of locomotion, no observations on crawling ^h

^a (Allcock, 2005).

^b (Allcock et al., 2003a).

^c (Allcock and Piertney, 2002).

^d (Allcock et al., 2003b).

^e (Daly and Rodhouse, 1994).

^f (Lu and Stranks, 1994).

^g (Collins and Villanueva, 2006).

^h (Vecchione et al., 1998).

ⁱ (Kubodera and Okutani, 1986).

Table 8

Diets based on studies of stomach contents from Antarctica octopods.

	<i>P. aequipapillae</i>	<i>P. turqueti</i>	<i>M. setebos</i>	<i>A. polymorpha</i>	<i>Cirroctopus</i> sp.
Amphipods	x ^{e,b}	x ^b	x ^b	x ^b	
Copepods					x ^{f,c}
Crustaceans		x ^a		x ^a	x ^d
Polychaetes		x ^{a,b}		x ^{a,b}	
Fish		x ^b	x ^b		
Gastropods		x ^a			
Bivalves		x ^a			
Octopods		x ^b			
Ophiuroids			x ^b		
Others	x ^{e,b}	x ^b	x		
Unknown	x ^{e,b}	x ^b	x ^b	x ^b	

^a (Daly, 1996).^b (Piatkowski et al., 2003).^c (Vecchione et al., 1998).^d Personal observations.^e Based solely on observations on *P. charcoti* collected between 90 and 130 m, as opposed to *P. aequipapillae*'s depth range of 190–750 m.^f Based on *Cirroctopus glacialis*. Unidentifiable crustacean fragments.

members of the genus, however, as *Pareledone charcoti*, a closely related congener of *P. aequipapillae*, apparently feeds almost exclusively on amphipods. Although this could indicate a specialisation on amphipod prey, octopod diet is often governed by prey abundance as well as preference (Ambrose, 1984), and could therefore reflect an encounter rate in shallow waters rather than selective foraging. Similarly, the high proportion of ophiuroids (62%) in the stomach of *M. setebos*, which also contained fish remains, is likely to reflect an abundance and ease of capture rather than ability and preference. *A. polymorpha* appears to specialise on amphipods and polychaetes, although a large proportion remains to be identified.

In a study on *Cirroctopus glacialis*, Vecchione et al. (1998) opened the stomachs of a few specimens, which turned out to either be empty or contain unidentifiable fragments of crustaceans. During the dissection of one specimen in this study, crustacean fragments and what appeared to be copepods were found in the digestive tract of a large individual. This is concordant with literature on the diet of other demersal cirrate octopods, which includes small, slow swimming prey, such as copepods, amphipods, and polychaetes (Collins and Villanueva, 2006).

Like in the morphological analysis, the species can be divided into groups, where *P. turqueti* appear as the true generalist, *M. setebos* as the opportunistic generalist, and *A. polymorpha* and *Cirroctopus* sp. as specialising generalists. Although the latter term may seem self-contradictory, it simply reflects a trend towards certain prey types within a generalist species' diet (Mather, 1991). *P. aequipapillae* cannot securely be grouped into either of these, as no dietary data has been published, and the depth range compared to *P. charcoti* is quite different.

Thus, considering both morphological traits and the apparent relative difficulties in prey capture faced by each species, *M. setebos* appears to be able to subdue prey mainly by brute force, *P. turqueti* seems to be dependent on venom to catch some of its prey, while *A. polymorpha* has the morphology and diet of a species adapted to the use of venom. *Cirroctopus* sp. does not depend on venom for prey capture, while *P. aequipapillae* remains inconclusive due to the lack of dietary information.

4. Discussion

4.1. Properties of Antarctic octopodid venom

As evident from the results from the venom analyses, Antarctic octopodid venom harbours a range of toxins, two of which have not previously been described from the Octopoda. Being the first investigation into the properties of the venom of the Antarctic Octopoda, the results from each assay are briefly discussed below.

4.1.1. Alkaline phosphatase

Previous activities reported for assays of venom ALP using the same substrate range from 0.2×10^{-3} to 0.4 U/mg venom protein in the spider *Loxosceles gaucho* (Sales and Santoro, 2008) and the viperid *Vipera ammodytes* (Risch et al., 2009), respectively, ALP activities in the PSG extracts were several orders of magnitude higher. There was also evidence for cold-adaptation, with consistently high activities at the enzymatically challenging temperature of 0 °C, actually exceeding those at 37 °C in all species except *P. aequipapillae*. In comparison, ALP activity from the photoreceptors of *E. cirrhosa* was shown to peak at 60 °C, with less than 10% maximum activity at 10 °C (Romero-Saravia and Hamdorf, 1983). This is similar to previously described venom ALP, where maximum activities occur between 45 and 55 °C and activity drops to 20–35% at 25 °C (Zhu et al., 2008). Rather, the high ALP activities at 0 °C suggest ALPs from Antarctic octopodid venom are "extreme" cold-adapted, a term referring to cold-adapted enzymes capable of functioning below sub-zero temperatures (Roach, 2002).

Combined, these results are strong indicators that ALP plays an important function in the prey capture: By causing an endogenous release of purines, venom ALP may immobilize prey by inducing hypotensive paralysis (Aird, 2002). Although ALP activity has never previously been described from octopod PSG, it is interesting to note that at least one of the constituents of Ctx, which caused hypotensive paralysis in crabs, was reported to be a glycoprotein, just like ALP (Ghiretti, 1960; McComb et al., 1979). Furthermore, the apparent absence of ALP in paralarval *Octopus* and

Japatella (Boucaud-Camou and Roper, 1995) PSG could indicate ontogenetic changes in the venom composition.

4.1.2. Acetylcholinesterase

By scavenging acetylcholine, AChE is thought to work synergistically with ALP in paralyzing prey through hypotension (Aird, 2002). Although there was probably very weak AChE activity in all extracts, including that from the *Cirroctopus* stomach tissue, inconsistent results within each assay repetition prevented any activities from being calculated with any degree of confidence. The presence of activity at seemingly approximately equal rates in both PSG and stomach, however, suggests that the AChE measured was present as housekeeping enzymes in both tissue-types, and is not present as an active ingredient in the secreted saliva. Another possibility is that there were unrecognized problems at some stage of the assay, as indicated by the variable absorbance values obtained from the controls.

4.1.3. Proteolytic activity

The presence of general proteolytic activity in the PSG extracts from the four assayed species is consistent with previous investigations (e.g. Fry et al., 2009; Grisley, 1993), confirming that proteases play an important part in prey handling in incirrate octopods. Fry et al. (2009) calculated that the molecular weights of the processed peptidase S1s from *O. kaurma* ranged from 25.25 to 26.89 kDa, while a molecular weight of 32 kDa was reported for a protease isolated from the saliva of *E. cirrhosa* (Grisley, 1993). These results fit well with the observed bands in the SDS-PAGE, which were found between 25 and 30 kDa in all but *M. setebos*, and at 35 kDa in PSG extracts.

Like ALP, there was also evidence for extreme cold-adaptation of proteases in the PSG extracts; except for *P. turqueti*, there was considerable proteolytic activity at 0 °C in all species, with *A. polymorpha* and *M. setebos* even showing almost twice the caseinolytic activity at 0 °C compared to 37 °C. It has been argued that because casein is not a natural substrate for the proteases contained in venom, not too much significance should be accredited to findings of suboptimal activity rates under near-natural conditions (Robertson and Delpierre, 1969). However, as the same substrate was used at both assay temperatures, the presence of near-equal activities at the two temperatures, or even a shift of an optimal enzymatic reaction temperature from near or above 37 °C to temperatures closer to 0 °C, still indicates an adaptation to colder environments. This is a very interesting finding, considering that most proteases from psychrophilic organisms have activity optima at considerably higher temperatures than their natural surroundings, often in the vicinity of 40 °C (Brier et al., 2007; Ditttrich, 1992a,b). As with ALP, proteases previously described from venoms show a very different thermal activity profile compared to that of Antarctic octopodids; typically peaking at 37–65 °C and usually showing a rapid drop in activity rates with reduced temperatures (Li et al., 2005a; Nakar et al., 1986; Rodrigues et al., 2000). The high activities at 0 °C is also unusual compared to enzymes described from temperate and tropical octopods where, although no data seems to be available on their performance at 0 °C and not all studies give

variation of activity around the optimum temperature, most seem to have maximum activity between 40 and 60 °C with a significant decrease in activity below 40 °C (D'Aniello et al., 1982; Fan et al., 2009; Feng et al., 2008).

Although the near lack of proteolytic activity in *P. turqueti* initially suggests no cold-adaptation is present, further indicating a less important role of proteases in the species, this is not necessarily so. While it cannot be deduced from the results of this study alone, findings of previous studies on octopod venom (Fry et al., 2009; Grisley, 1993; Morishita, 1974) suggest that several proteases may be present in the saliva of Antarctica octopodids, including *P. turqueti*. This means that, although the protease (s) responsible for the proteolytic activity observed in the assay may not be cold-adapted in *P. turqueti*, it does not exclude the possibility of other important cold-adapted proteases being present. The low proteolytic activity of the venom can thus be explained by a high substrate specificity of the venom proteases which cannot be realized with casein as substrate. Another possibility is the presence of protease inhibitors, which are common constituents in snake venoms (Lu et al., 2008). However, the low proteolytic activity level at 0 °C compared to 37 °C does indicate that there are probably differences between the proteases in the venom of *P. turqueti* compared to that of the remaining incirrates.

4.1.4. Secreted Phospholipase A₂

Although present, none of the detected sPLA₂ activities of the PSG extracts were especially high, with the maximum activity being that of *P. aequipapillae* at 1.0511 U/mg. In comparison to the positive control, bee venom PLA₂ (287.5 U/mg) or crude venoms known to contain potent PLA₂s, such as from the elapid *Acanthophiphis rugosus* (140.2 U/mg) (Wickramaratna et al., 2003), it may seem that sPLA₂ is not a particularly important component of the assayed venoms. However, the enzymatic activity of PLA₂ is not necessarily correlated with toxicity (Rosenberg, 1990). Ecarpholin S, for example, a PLA₂-type toxin found in the venom of the viperid *Echis carinatus*, possesses very weak specific enzymatic activity (0.1 U/mg) but is still a potent myotoxin (Zhou et al., 2008). Because logistic constraints prevented the assay from being carried out at 0 °C, it was not found whether or not the sPLA₂ present showed any signs of adaptation to functioning under low temperatures.

The presence of sPLA₂ in the venom of Antarctic octopods is in itself an interesting find, however, as it has implications for the understanding of the evolutionary history of cephalopod toxins. While transcripts coding for PLA₂ have been found in the PSG of the cuttlefish *Sepia latimanus*, none could be detected in neither *O. kaurma* nor *H. maculosa* (Fry et al., 2009), which are both members of the octopodid sub-family Octopodinae. As the venom-producing species included here are all members of Eleledoninae, another octopodid sub-family, the positive sPLA₂ assay results suggest three possible scenarios: A basal cephalopod sPLA₂ radiation with a secondary loss in Octopodinae; an independent recruitment in the Octopoda with secondary loss in the Octopodinae; or an independent recruitment at some stage of the evolutionary history of Antarctic eleledonine octopods after the divergence from

Octopodinae but prior to their radiation approximately 50 million years ago (Strugnell et al., 2008). Unfortunately, the results obtained here reveal nothing about which of these scenarios is the most likely. Although the cuttlefish PLA₂ was unusually large (22.3 kDa) (Fry et al., 2009), and venom PLA₂ known from other molluscs is much smaller (13.6 kDa) (McIntosh et al., 1995), bands were observed in both regions on the SDS-PAGE gel (Fig. 3). These latter bands, however, also match the sizes of two of the novel peptides discovered from *O. kaurna* (Fry et al., 2009), which functions remain unknown. It will therefore be interesting to follow future studies on the structure and size of the proteins responsible for the sPLA₂ activities observed, particularly if a recruitment independent of that in cuttlefish is the case.

4.1.5. Haemolytic activity

PSG extracts from three species exhibited haemolytic activity on mammalian red blood cells. Assuming salivary protein concentrations estimated previously (Grisley et al., 1999) and calculating percent haemolysis per mg protein, the activities of *M. setebos* and *P. aequipapillae* were slightly lower, but comparable, to that reported from the saliva of *E. cirrhosa* (Key et al., 2002). Key et al. (2002) found the presence of haemolytic activity in octopus saliva difficult to explain, but suggested it was due to components that generally caused an increase in the permeability of cell membranes, thereby enabling a higher efficiency of secreted digestive enzymes. An eruption of cell membranes would also increase the amount of substrate available to the salivary ALP, thereby playing a part in the hypotensive actions of the venom. Although the increase in membrane permeability could be due to the cytolytic action of one or several different toxins (Harvey, 1990), one component able to destabilize and disrupt cell membranes detected in this study is sPLA₂, which may have this as a pharmacological side-effect (Rosenberg, 1990). It may also be that the haemolysis observed was primarily caused by an unknown component, which would explain the presence of sPLA₂ but absence of haemolytic activity in the PSG extracts of *P. turqueti*. However, as haemolytic agents are extremely heterogeneous, with no common mechanism of action and molecular weights ranging from 2 to 290 kDa (Harvey, 1990), this remains at a purely speculative level.

4.1.6. The effect of small sample sizes

The fact that only a few specimens from each species could be used leaves several factors that may influence the composition of venom unaccounted for, including size, sex, condition, and time allowed for regeneration of toxins (e.g. Boevé et al., 1995; Herzig et al., 2008). The main implications of this are that negative assay results do not exclude the possibility of the activity being present at another life stage or in another specimen, and that the activity levels cannot be compared between species with any real degree of certainty. Although the positive results may in theory only be applicable to individuals of a certain size and sex (which is also the case in studies where large samples have been pooled), and while one cannot draw conclusions based on the difference in assay activities between species, the assay results do provide useful information on what

activities are present in each species. Similarly, the fact that only one sample (extract; pooled or not) could be assayed for each species means the variation in enzymatic function is unaccounted for, leaving a potentially high degree of uncertainty as to the relative activity rates between 0 °C and 37 °C. The results nevertheless show a presence or absence of activity at 0 °C and whether this activity is comparable or potentially higher than that at 37 °C.

4.2. The importance of venom

As mentioned introductorily, venom plays an important part in the trophic ecologies of many temperate and tropical octopod species, both in the immobilization of prey and processing it prior to ingestion. This also seems to be the case for Antarctic eledonines, as indicated by the range of activities common to animal venoms exhibited by the PSG extracts, particularly considering the apparent need for selection in the maintenance of toxin functional properties (Li et al., 2005b; Ohno et al., 2002).

In addition to such maintenance-level selection pressure, the functional cold-adaptations of ALP and proteases indicate such a central role of these enzymes that they may have undergone adaptive evolution. One such role might be as a way of compensating for the low metabolic rate in cold environments: *P. charcoti*, a close Southern Ocean endemic relative of the incirrate species included here, shows no evidence of metabolic cold-adaptation and has an at least as low metabolism as that extrapolated for *E. cirrhosa* at 0 °C (Daly and Peck, 2000). By increasing the efficiency of their salivary toxins, Antarctic incirrates may therefore be compensating for the low energy level available to foraging by using venom to rapidly immobilize prey. Similarly, as octopod proteases have been found to aid in the ingestion of crustacean prey by facilitating musculo-skeletal detachment (Grisley, 1993; Nixon, 1984), cold-adapted salivary proteases could greatly increase not just the efficiency of the energy spent on feeding but also the amount of food obtained from each prey item.

4.3. Venom, morphology and diet

Although venom undoubtedly plays an important part in the prey capture and processing of Antarctic eledonines, no obvious venom-adaptations to differences in diet or morphology were apparent from the results of the enzymatic and haemolytic assays. While this may be due to the opportunistic generalist nature of octopodid diets preventing any longer durations of selection from prey preference to occur, it is more likely to be due to limitations of the activity assays; the use of a single substrate tells nothing of an enzyme's various substrate affinities and hence possible adaptations to specific prey. For example, like the potency of Ctx differs between various crustaceans (Ghiretti, 1960), the enzymatic toxins assayed here may have different reaction rates in different prey species and so be adapted to particular diets. Conversely, the interspecific differences in the assay results may reflect functional adaptations to different roles or prey types, although they do not indicate for what role or to which prey.

Another possibility, of course, is the presence of toxins other than those included here, such as the neuropeptides described from both Eledoninae (Erspamer and Anastasi, 1962) and Octopodinae (Fry et al., 2009; Kanda et al., 2003). This may be the case for *A. polymorpha*, which venom appears from the assay results to be typical ancestral venom, devoid of any particular specialisations. However, compared to the remaining incirrates, *A. polymorpha* possesses several unusual morphological features, including very large PSG, a sharp narrow beak, and a small buccal mass. Combined, these traits appear characteristic of a species relying heavily on venom: While large PSG by themselves are indicators of venom being important (Gibbs and Greenaway, 1978), the small buccal mass leaves little room for powerful musculature enabling crushing force of the beak, which rather seems adapted to piercing than taking chunks out of prey. An increased importance of venom through the specialisation in drilling holes in shelled prey also seems unlikely considering the size of the buccal mass, as this would then contain an extra organ and likely to be larger due to the muscular actions required for the sustained radular movement (Messenger and Young, 1999).

While several authors have commented on this unusual set of traits, suggesting it may be an adaptation to hunting in the water column (Allcock et al., 2003a; Daly and Rodhouse, 1994), no evidence for this could be found in the dietary data, as *A. polymorpha* seems to prey mainly on amphipods and polychaetes (Piatkowski et al., 2003). However, considering the size of many Antarctic polychaetes, in the trawl commonly exceeding 14 cm by 2.5 cm, and which often have powerful jaws, *A. polymorpha* may have adapted to the use of venom to subdue large polychaete prey. In contrast, the morphology of the other species known to feed on polychaetes, *P. turqueti*, is like that of other octopodids (Allcock and Piertney, 2002), suggesting it is adapted to a more generalist diet. Being of similar sizes and often appearing sympatrically, ecological niche partitioning between the two species may have resulted in a trophic shift in *A. polymorpha* due to, or followed by, adaptive toxin evolution and associated specialisation of morphology. If so, this would then represent the opposite scenario to that of *Cirroctopus* which is highly a gelatinous body, docile locomotion, and lack of venom apparatus could well be linked to its use of cirri in the feeding upon small crustaceans such as copepods. In addition, the remaining two species of *Adelieledone*, *A. adeliana* and *A. piatkowski*, may be particularly interesting for future Antarctic octopod venom research, as this morphological adaptation must have occurred prior to the radiation of *Adelieledone* (Allcock et al., 2003a).

4.4. Conclusions

This study provides the first ever insights into the venomous function of Antarctic Octopodidae. From the range of toxic activities found in all venoms, as well as evidence for cold-adaptation in at least one venom component in all incirrates studied, it is evident that venom plays an important role in the ecology of Antarctic octopodids. Furthermore, although no clear link connecting diet, morphology and venom properties was found due to

limitations of reliable prey-preference data, indications that such a relationship exists are apparent from the comparative morphologies of *Cirroctopus*, *Adelieledone*, *Megaleledone*, and *Pareledone*. More research is needed to confirm this, however, including dietary studies and detailed characterization of venom components. Considering the issues raised by the results regarding both Cephalopod venom evolution and functional adaptation of psychrophilic toxin proteins, it seems that the possibilities for further research into the field of Antarctic cephalopod venoms are vast. Future research and progression towards gaining a picture of both the evolutionary role of venom and its means of functioning in this unique but inhospitable environment is therefore likely to generate some very interesting results, in terms of both molecular adaptive evolution and potential pharmaceutical value.

Acknowledgements

Funding for this project was provided by the Australian Antarctic Division and the Herman Slade Foundation. We would also like to express our sincere thanks and gratitude to the Australian Antarctic Division for providing sea-time aboard *RV Aurora Australis*, the crew onboard *RV Aurora Australis* for bringing up countless trawl catches, and to voyage leader Dr Martin Riddle and deputy voyage leader Ms Sarah Robinson for a productive research cruise.

Also big thanks to the NTNU student health office, Gloshaugen, Trondheim, for input on the preparation of red blood cells used in the haemolytic assay.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

- Aird, S.D., 2002. Ophidian envenomation strategies and the role of purines. *Toxicon* 40, 335–393.
- Allcock, A.L., 2005. On the confusion surrounding *Pareledone charcoti* (Joubin, 1905) (Cephalopoda: Octopodidae): endemic radiation in the Southern ocean. *Zool. J. Linn. Soc.* 143, 75–108.
- Allcock, A.L., Brierley, A.S., Thorpe, J.P., Rodhouse, P.G., 1997. Restricted gene flow and evolutionary divergence between geographically separated populations of the Antarctic octopus *Pareledone turqueti*. *Mar. Biol.* 129, 97–102.
- Allcock, A.L., Piatkowski, U., Rodhouse, P., Thorpe, J., 2001. A study on octopodids from the eastern Weddell Sea, Antarctica. *Polar Biol.* 24, 832–838.
- Allcock, A.L., Hochberg, F.G., Rodhouse, P.G.K., Thorpe, J.P., 2003a. *Adelieledone*, a new genus of octopodid from the Southern Ocean. *Antarct. Sci.* 15, 415–424.
- Allcock, A.L., Hochberg, F.G., Stranks, T.N., 2003b. Re-evaluation of *Graneledone setebos* (Cephalopoda: Octopodidae) and allocation to the genus *Megaleledone*. *J. Mar. Biolog. Assoc. U.K.* 83, 319–328.
- Allcock, A.L., Lockhart, S., Ellingsen, K., Mooi, R., de Broyer, C., 2003c. Introduction to work at sea. *Ber. Polar Meeresforsch.* 470, 91–94.
- Allcock, A.L., Strugnell, J., Prodöhl, P., Piatkowski, U., Vecchione, M., 2007. A new species of *Pareledone* (Cephalopoda: Octopodidae) from Antarctic Peninsula waters. *Polar Biol.* 30, 883–893.
- Allcock, A.L., Piertney, S., 2002. Evolutionary relationships of Southern Ocean Octopodidae (Cephalopoda: Octopa) and a new diagnosis of *Pareledone*. *Mar. Biol.* 140, 129–135.
- Ambrose, R.F., 1984. Food preferences, prey availability, and the diet of *Octopus bimaculatus* Verrill. *J. Exp. Mar. Biol. Ecol.* 77, 29–44.
- Ambrose, R.F., Nelson, B.V., 1983. Predation by *Octopus vulgaris* in the Mediterranean. *Mar. Ecol.* 4, 251–261.

- Berkov, A., Rodríguez, N., Centeno, P., 2008. Convergent evolution in the antennae of a cerambycid beetle, *Onychocerus albitarsis*, and the sting of a scorpion. *Naturwissenschaften* 95, 257–261.
- Boevé, J.-L., Kuhn-Nentwig, L., Keller, S., Nentwig, W., 1995. Quantity and quality of venom released by a spider (*Cupiennius salei*, Ctenidae). *Toxicon* 33, 1347–1357.
- Boucaud-Camou, E., Roper, C.F.E., 1995. Digestive enzymes in paralarval cephalopods. *Bull. Mar. Sci.* 57, 313–327.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Brier, S., Maria, G., Carginale, V., Capasso, A., Wu, Y., Taylor, R.M., Borotto, N.B., Capasso, C., Engen, J.R., 2007. Purification and characterization of pepsins A₁ and A₂ from the Antarctic rock cod *Trematomus bernacchii*. *FEBS J.* 274, 6152–6166.
- Cavazzoni, E., Lister, B., Sargent, P., Schibler, A., 2008. Blue-ringed octopus (*Hapalochlaena* sp.) envenomation of a 4-year-old boy: a case report. *Clin. Toxicol.* 46, 760–761.
- Chen, D., Kini, R.M., Yuen, R., Khoo, H.E., 1997. Haemolytic activity of stonustoxin from stonefish (*Synanceja horrida*) venom: pore formation and the role of cationic amino acid residues. *Biochem. J.* 325, 685–691.
- Chetty, N., Du, A., Hodgson, W.C., Winkel, K., Fry, B.G., 2004. The in vitro neuromuscular activity of Indo-Pacific sea-snake venoms: efficacy of two commercially available antivenoms. *Toxicon* 44, 193–200.
- Clarke, A., 2008. Antarctic marine benthic diversity: patterns and processes. *J. Exp. Mar. Biol. Ecol.* 366, 48–55.
- Collins, M.A., Rodhouse, P.G.K., 2006. Southern ocean cephalopods. *Adv. Mar. Biol.* 50, 191–265.
- Collins, M.A., Villanueva, R., 2006. Taxonomy, ecology and behaviour of the cirrate octopods. *Oceanogr. Mar. Biol.* 44, 277–322.
- Costa, J.n.d.O., Fonseca, K.C., Neves Mamede, C.C., Beletti, M.E., Santos-Filho, N.A., Soares, A.M., Arantes, E.C., Hirayama, S.N.S., Selistre-Araújo, H.S., Fonseca, F., Henrique-Silva, F.v., Penha-Silva, N., Oliveira, F.b.d., 2010. Bhaltermin: functional and structural characterization of a new thrombin-like enzyme from *Bothrops alternatus* snake venom. *Toxicon* 55, 1365–1377.
- Cousin, X., Bon, S., Duval, N., Massoulie, J., Bon, C., 1996. Cloning and expression of Acetylcholinesterase from *Bungarus fasciatus* venom. A new type of COOH-terminal domain; involvement of a positively charged residue in the peripheral site. *J. Biol. Chem.* 271, 15099–15108.
- D'Aniello, A., Hakimi, J., Cacace, G.M., Ceccarini, C., 1982. The purification and characterization of {alpha}-L-Fucosidase from the hepatopancreas of *Octopus vulgaris*. *J. Biochem.* 91, 1073–1080.
- Daly, H.I., 1996. Ecology of the Antarctic Octopus *Pareledone* from the Scotia Sea. University of Aberdeen.
- Daly, H.I., Peck, L.S., 2000. Energy balance and cold adaptation in the octopus *Pareledone carcoti*. *J. Exp. Mar. Biol. Ecol.* 245, 197–214.
- Daly, H.I., Rodhouse, P.G., 1994. Comparative morphology of two sympatric *Pareledone* species from South Georgia. *Antarct. Sci.* 6, 163–169.
- Devaraja, S., Nagaraju, S., Mahadeswaraswamy, Y.H., Girish, K.S., Kemparaju, K., 2008. A low molecular weight serine protease: purification and characterization from *Hippasa agelenoides* (funnel web) spider venom gland extract. *Toxicon* 52, 130–138.
- Dittrich, B., 1992a. Thermal acclimation and kinetics of a trypsin-like protease in eucarid crustaceans. *J. Comp. Physiol. B. Biochem. Syst. Environ. Physiol.* 162, 38–46.
- Dittrich, B.U., 1992b. Life under extreme conditions: aspects of evolutionary adaptation to temperature in crustacean proteases. *Polar Biol.* 12, 269–274.
- Duda, T.F., Palumbi, S.R., 1999. Molecular genetics of ecological diversification: duplication and rapid evolution of toxin genes of the venomous gastropod *Conus*. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6820–6823.
- Erspamer, V., Anastasi, A., 1962. Structure and pharmacological actions of eledoisin, the active endecapeptide of the posterior salivary glands of *Eledone*. *Cell. Mol. Life Sci.* 18, 58–59.
- Erspamer, V., Asero, B., 1953. Isolation of enteramine from extracts of posterior salivary glands of *Octopus vulgaris* and of *Discoglossus pictus* skin. *J. Biol. Chem.* 200, 311–318.
- Fan, T., Li, M., Wang, J., Yang, L., Cong, R., 2009. Purification and characterization of phenoloxidase from *Octopus ocellatus*. *Acta Biochim. Biophys. Sin.* 41, 865–872.
- Farid, T.M., Tu, A.T., El-Asmar, M.F., 1989. Characterization of cerastobin, a thrombin-like enzyme from the venom of *Cerastes vipera* (Sahara sand viper). *Biochemistry* 28, 371–377.
- Feng, L., Gao, R., Gopalakrishnakone, P., 2008. Isolation and characterization of a hyaluronidase from the venom of Chinese red scorpion *Buthus martensi*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 148, 250–257.
- Fiorito, G., Gherardi, F., 1999. Prey-handling behaviour of *Octopus vulgaris* (Mollusca, Cephalopoda) on Bivalve preys. *Behav. Processes* 46, 75–88.
- Fry, B.G., 2005. From genome to “venome”: molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. *Genome Res.* 15, 403–420.
- Fry, B.G., Wüster, W., Ramjan, S.F.R., Jackson, T., Martelli, P., Kini, R.M., 2003. Analysis of Colubroidea snake venoms by liquid chromatography with mass spectrometry: evolutionary and toxicological implications. *Rapid Commun. Mass Spectrom.* 17, 2047–2062.
- Fry, B.G., Wickramaratana, J.C., Lemme, S., Beuve, A., Garbers, D., Hodgson, W.C., Alewood, P., 2005. Novel natriuretic peptides from the venom of the inland taipan (*Oxyuranus microlepidotus*): isolation, chemical and biological characterisation. *Biochem. Biophys. Res. Commun.* 327, 1011–1015.
- Fry, B.G., Vidal, N., Norman, J.A., Vonk, F.J., Scheib, H., Ramjan, S.F.R., Kuruppu, S., Fung, K., Blair Hedges, S., Richardson, M.K., Hodgson, W.C., Ignjatovic, V., Summerhayes, R., Kochva, E., 2006. Early evolution of the venom system in lizards and snakes. *Nature* 439, 584–588.
- Fry, B.G., Scheib, H., van der Weerd, L., Young, B., McNaughtan, J., Ramjan, S.F.R., Vidal, N., Poelmann, R.E., Norman, J.A., 2008. Evolution of an Arsenal: structural and functional diversification of the venom system in the advanced snakes (Caenophidia). *Mol. Cell Proteomics* 7, 215–246.
- Fry, B.G., Roelants, K., Norman, J., 2009. Tentacles of venom: toxic protein convergence in the Kingdom Animalia. *J. Mol. Evol.* 68, 311–321.
- Fry, B.G., Wüster, W., 2004. Assembling an arsenal: origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences. *Mol. Biol. Evol.* 21, 870–883.
- Ghiretti, F., 1959. Cephalotoxin: the crab-paralysing agent of the posterior salivary glands of Cephalopods. *Nature* 183, 1192–1193.
- Ghiretti, F., 1960. Toxicity of octopus saliva against crustacea. *Ann. N.Y. Acad. Sci.* 90, 726–741.
- Gibbs, P.J., Greenaway, P., 1978. Histological structure of the posterior salivary glands in the blue ringed octopus *Hapalochlaena maculosa* Hoyle. *Toxicon* 16, 59–70.
- Goyffon, M., 2002. The venomous function. In: Ménez, A. (Ed.), *Perspectives in Molecular Toxinology*. John Wiley and Sons, West Sussex, pp. 423–433.
- Grisley, M.S., 1993. Separation and partial characterization of salivary enzymes expressed during prey handling in the octopus *Eledone cirrhosa*. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 105, 183–192.
- Grisley, M.S., Boyle, P.R., 1987. Bioassay and proteolytic activity of digestive enzymes from octopus saliva. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 88, 1117–1123.
- Grisley, M.S., Boyle, P.R., Key, L.N., 1996. Eye puncture as a route of entry for saliva during predation on crabs by the octopus *Eledone cirrhosa* (Lamarck). *J. Exp. Mar. Biol. Ecol.* 202, 225–237.
- Grisley, M.S., Boyle, P.R., Pierce, G.J., Key, L.N., 1999. Factors affecting prey handling in lesser octopus (*Eledone cirrhosa*) feeding on crabs (*Carcinus maenas*). *J. Mar. Biolog. Assoc. U.K.* 79, 1085–1090.
- Grubert, M.A., Wadley, V.A., White, R.W.G., 1999. Diet and feeding strategy of *Octopus maorum* in southeast Tasmania. *Bull. Mar. Sci.* 65, 441–451.
- Guimarães, L.H., Terenzi, H.F., Jorge, J.A., Polizeli, M.L., 2001. Thermostable conidial and mycelial alkaline phosphatases from the thermophilic fungus *Scytalidium thermophilum*. *J. Ind. Microbiol. Biotechnol.* 27, 265–270.
- Gusmani, L., Avian, M., Galil, B., Patriarca, P., Rottini, G., 1997. Biologically active peptides in the venom of the jellyfish *Rhopilema nomadica*. *Toxicon* 35, 637–648.
- Harvey, A.L., 1990. Cytolytic toxins. In: Shier, T.W., Mebs, D. (Eds.), *Handbook of Toxinology*. Marcel Dekker, New York, pp. 1–66.
- Heatwole, H., Poran, N.S., 1995. Resistances of sympatric and Allopatric Eels to Sea snake venoms. *Copeia* 1995, 136–147.
- Heitz, J.R., Norment, B.R., 1974. Characteristics of an alkaline phosphatase activity in brown recluse venom. *Toxicon* 12, 181–187.
- Henze, M., 1913. *p*-Oxyphanylæthylamin, das Speicheldrusengift der Cephalopoden. *Hoppe-Seyler's Z. Physiol. Chem.* 87, 51–58.
- Hernandez-Oliveira, S., Toyama, M., Toyama, D., Marangoni, S., Hyslop, S., Rodrigues-Simioni, L., 2005. Biochemical, pharmacological and structural characterization of a new PLA₂ from *Crotalus durissus terrificus* (South American rattlesnake) venom. *Protein J.* 24, 233–242.
- Herzig, V., Khalife, A.A., Chong, Y., Isbister, G.K., Currie, B.J., Churchill, T.B., Horner, S., Escoubas, P., Nicholson, G.M., Hodgson, W.C., 2008.

- Intersexual variations in Northern (*Missulena pruinosa*) and Eastern (*M. bradleyi*) mouse spider venom. *Toxicon* 51, 1167–1177.
- Johnson, A.J., Kline, D.L., Alkjaersig, N., 1969. Assay methods and standard preparations for plasmin, plasminogen and urokinase in purified systems, 1967–1968. *Thromb. Diath. Haemorrh* 21, 259–272.
- de Jong, M.C.J.M., Kawamoto, F., Bleumink, E., Kloosterhuis, A.J., Meijer, G. T., 1982. A comparative study of the spicule venom of Euproctis caterpillars. *Toxicon* 20, 477–485.
- Kanda, A., Iwakoshi-Ukena, E., Takuwa-Kuroda, K., Minakata, H., 2003. Isolation and characterization of novel tachykinins from the posterior salivary gland of the common octopus *Octopus vulgaris*. *Peptides* 24, 35–43.
- Key, L.N., Boyle, P.R., Jaspars, M., 2002. Novel activities of saliva from the octopus *Eledone cirrhosa* (Mollusca: Cephalopoda). *Toxicon* 40, 677–683.
- Kordis, D., Gubensek, F., 2000. Adaptive evolution of animal toxin multigene families. *Gene* 261, 43–52.
- Kubodera, T., Okutani, T., 1986. New and rare Cephalopods from the Antarctic waters. *Mem. Natl. Inst. Polar Res.*, 129–143. Spec. Issue.
- Lewis, J.G.E., 1981. *The Biology of Crustaceans*. Cambridge University Press, Cambridge.
- Lewis, R.J., Garcia, M.L., 2003. Therapeutic potential of venom peptides. *Nat. Rev. Drug Discov.* 2, 790–802.
- Li, C., Yu, H., Liu, S., Xing, R., Guo, Z., Li, P., 2005a. Factors affecting the protease activity of venom from jellyfish *Rhopilema esculentum* Kishinouye. *Bioorg. Med. Chem. Lett.* 15, 5370–5374.
- Li, M., Fry, B.G., Kini, R.M., 2005b. Putting the brakes on snake venom evolution: the unique molecular evolutionary patterns of *Aipysurus eydouxi* (Marbled Sea Snake) Phospholipase A₂ toxins. *Mol. Biol. Evol.* 22, 934–941.
- Liu, S., Sun, M.-Z., Greenaway, F.T., 2006. A novel plasminogen activator from *Agkistrodon blomhoffii* *ussurensis* venom (ABUSV-PA): purification and characterization. *Biochem. Biophys. Res. Commun.* 348, 1279–1287.
- Liu, S., Sun, M.-Z., Sun, C., Zhao, B., Greenaway, F.T., Zheng, Q., 2008. A novel serine protease from the snake venom of *Agkistrodon blomhoffii* *ussurensis*. *Toxicon* 52, 760–768.
- Lu, J., Yang, H., Yu, H., Gao, W., Lai, R., Liu, J., Liang, X., 2008. A novel serine protease inhibitor from *Bungarus fasciatus* venom. *Peptides* 29, 369–374.
- Lu, C.C., Stranks, T.N., 1994. Synopsis of *Pareledone* and *Megaleledone* species, with description of two new species from East Antarctica (Cephalopoda: Octopodidae). *Mem. Mus. Vic.* 54, 221–242.
- Magalhães, M.R., da Silva Jr., N.J., Ulhoa, C.J., 2008. A hyaluronidase from *Potamotrygon motoro* (freshwater stingrays) venom: isolation and characterization. *Toxicon* 51, 1060–1067.
- Mather, J.A., 1991. Foraging, feeding and prey remains in middens of juvenile *Octopus vulgaris* (Mollusca: Cephalopoda). *J. Zool.* 224, 27–39.
- Matsui, T., Fujimura, Y., Titani, K., 2000. Snake venom proteases affecting hemostasis and thrombolysis. *Biochim. Biophys. Acta Protein Struct. Mol. Enzym.* 1477, 146–156.
- McComb, R.B., Bowers, G.N.J., Posen, S., 1979. *Alkaline Phosphatase*. Plenum Press, New York.
- McDonald, N.M., Cottrell, G.A., 1972. Purification and mode of action of toxin from *Eledone cirrhosa*. *Comp. Gen. Pharmacol.* 3, 243–248.
- McIntosh, J.M., Ghomashchi, F., Gelb, M.H., Dooley, D.J., Stoehr, S.J., Giordani, A.B., Naisbitt, S.R., Olivera, B.M., 1995. Conodipine-M, a novel Phospholipase A(2) isolated from the venom of the marine snail *Conus magus*. *J. Biol. Chem.* 270, 3518–3526.
- Messenger, J.B., Young, J.Z., 1999. The radular apparatus of cephalopods. *Philos. Trans. R. Soc. Lond., B. Biol. Sci.* 354, 161–182.
- Morey, S.S., Kiran, K.M., Gadag, J.R., 2006. Purification and properties of hyaluronidase from *Palamneus gravimanus* (Indian black scorpion) venom. *Toxicon* 47, 188–195.
- Morishita, T., 1974. Participation in digestion by the proteolytic enzymes of the posterior salivary gland in *Octopus*-II Isolation and purification of the proteolytic enzymes from the posterior salivary gland. *Nippon Suisan Gakkai Shi* 40, 601–607.
- Nakar, O., Ovadia, M., Kochva, E., 1986. Isolation and characterization of a proteolytic factor from the venom of *Vipera palaestinae*. *Toxicon* 24, 293–304.
- Nixon, M., 1984. Is there external digestion by *Octopus*? *J. Zool.* 202, 441–447.
- Ohno, M., Ogawa, T., Oda-Ueda, N., Chijiwa, T., Hattori, S., 2002. Accelerated and regional evolution of snake venom gland isozymes. In: Ménez, A. (Ed.), *Perspectives in Molecular Toxinology*. Wiley and Sons, West Sussex, pp. 387–400.
- Pandya, B.V., Budzynski, A.Z., 1984. Anticoagulant proteases from western diamondback rattlesnake (*Crotalus atrox*) venom. *Biochemistry* 23, 460–470.
- Piatkowski, U., Allcock, A.L., Vecchione, M., 2003. Cephalopod diversity and ecology. *Berichte-zur-Polar-und-Meerforschung* 470, 32–38.
- Pilson, M.E.Q., Taylor, P.B., 1961. Hole drilling by octopus. *Science* 134, 1366–1368.
- Poh, C.H., Yuen, R., Chung, M.C.M., Khoo, H.E., 1992. Purification and partial characterization of hyaluronidase from stonefish (*Synanceja horrida*) venom. *Comp. Biochem. Physiol. Part B. Comp. Biochem.* 101, 159–163.
- Quinn, D.M., 1987. Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states. *Chem. Rev.* 87, 955–979.
- Risch, M., Georgieva, D.N., von Bergen, M., Jehmlich, N., Genov, N., Arni, R.K., Betzel, C., 2009. Snake venomomics of the Siamese Russell's viper (*Daboia russelli siamensis*) – relation to pharmacological activities. *J. Proteomics* 72, 256–269.
- Roach, J.C., 2002. A clade of trypsins found in cold-adapted fish. *Proteins: Struct. Funct. Genet.* 47, 31–44.
- Robertson, S.S.D., Delpierre, G.R., 1969. Studies on African snake venoms - IV Some enzymatic activities in the venom of the boomslang *Dispholidus typus*. *Toxicon* 7, 189–194.
- Rodrigues, V.M., Soares, A.M., Guerra-S., R., Rodrigues, V., Fontes, M.R.M., Giglio, J.R., 2000. Structural and functional characterization of Neuwiedea, a nonhemorrhagic fibrin(ogen)olytic metalloprotease from *Bothrops neuwiedi* snake venom. *Arch. Biochem. Biophys.* 381, 213–224.
- Rodrigues, M.C.A., Guimarães, L.H.S., Liberato, J.L., Polizeli, M.D.L.T.D.M., dos Santos, W.F., 2006. Acid and alkaline phosphatase activities of a fraction isolated from *Parawixia bistriata* spider venom. *Toxicon* 47, 854–858.
- Romero-Saravia, O., Hamdorf, K., 1983. Alkaline phosphatase. A dominant enzyme of microvillus structure of cephalopod photoreceptors. *Biochim. Biophys. Acta Biomembr.* 729, 90–96.
- Rosenberg, P., 1990. *Phospholipases*. In: Shier, T.W., Mebs, D. (Eds.), *Handbook of Toxinology*. Marcel Dekker, New York, pp. 67–278.
- Runham, N.W., Bailey, C.J., Carr, M., Evans, C.A., Malham, S., 1997. Hole drilling in crab and gastropod shells by *Eledone cirrhosa* (Lamarck, 1798). *Sci. Mar.* 61, 67–76.
- Sales, P.B.V., Santoro, M.L., 2008. Nucleotidase and DNase activities in Brazilian snake venoms. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 147, 85–95.
- Sazima, I., Bastos-de-Almeida, L., 2006. The bird kraken: octopus preys on a sea bird at an oceanic island in the tropical west Atlantic. *J. Mar. Biol. Assoc. U.K., Biodiv. Rec.*
- Sheumack, D.D., Howden, M.E.H., Spence, I., Quinn, R.J., 1978. Maculotoxin: a neurotoxin from the venom glands of the octopus *Haplochlachena maculosa* identified as tetrodotoxin. *Science* 199, 188–189.
- Songdahl, J.H., Shapiro, B.I., 1974. Purification and composition of a toxin from the posterior salivary gland of *Octopus dofleini*. *Toxicon* 12, 109–112.
- Strugnell, J., Norman, M., Jackson, J., Drummond, A.J., Cooper, A., 2005. Molecular phylogeny of coleoid cephalopods (Mollusca: Cephalopoda) using a multigene approach; the effect of data partitioning on resolving phylogenies in a Bayesian framework. *Mol. Phylogenet. Evol.* 37, 426–441.
- Strugnell, J., Rogers, A.D., Prodöhl, P.A., Collins, M.A., Allcock, A.L., 2008. The thermohaline expressway: the Southern Ocean as a centre of origin for deep-sea octopuses. *Cladistics* 24, 853–860.
- Sulkowski, E., Björk, W., Laskowski, M., 1963. A specific and nonspecific alkaline monophosphatase in the venom of *Bothrops atrox* and their occurrence in the purified venom phosphodiesterase. *J. Biol. Chem.* 238, 2477–2486.
- Sun, M.-Z., Liu, S., Yang, F., Greenaway, F.T., Xu, Y., 2009. A novel phospholipase A₂ from *Agkistrodon blomhoffii* *ussurensis* venom: purification, proteomic, functional and structural characterizations. *Biochimie* 91, 558–567.
- Tracy, R.P., Alport, E.C., Yunginger, J.W., Homburger, H.A., 1980. An immuno-enzyme assay for quantitation of human IgG antibodies to honeybee venom phospholipase A₂. *J. Immunol. Methods* 32, 185–193.
- Tu, A.T., Chua, A., 1966. Acid and alkaline phosphomonoesterase activities in snake venoms. *Comp. Biochem. Physiol.* 17, 297–307.
- Undheim, E.A.B., Norman, J.A., Thoen, H.H., Fry, B.G., 2010. Identification of Southern Ocean octopus samples using mtCOL. *CR Biol.* 333, 395–404.
- Valério, A.A., Corradini, A.C., Panunto, P.C., Mello, S.M., Hyslop, S., 2002. Purification and characterization of a phosphodiesterase from *Bothrops alternatus* snake venom. *J. Protein Chem.* 21, 495–503.
- Vecchione, M.M., Piatkowski, M.U., Allcock, M.A., 1998. Biology of the cirrate octopus *Grimpoteuthis glacialis* (Cephalopoda: Opisthoteuthidae) in the South Shetland islands, Antarctica. *S. Afr. J. Mar. Sci.* 20, 421–428.
- Vetter, R.S., 2008. Medical aspects of spider bites. *Annu. Rev. Entomol.* 53, 409.

- Wickramaratna, J.C., Fry, B.G., Aguilar, M.-I., Kini, R.M., Hodgson, W.C., 2003. Isolation and pharmacological characterization of a phospholipase A₂ myotoxin from the venom of the Irian Jayan death adder (*Acanthophis rugosus*). *Br. J. Pharmacol.* 138, 333–342.
- Yotsu-Yamashita, M., Mebs, D., Flachsenberger, W., 2007. Distribution of tetrodotoxin in the body of the blue-ringed octopus (*Hapalochlaena maculosa*). *Toxicon* 49, 410–412.
- Zhou, X., Tan, T.-C., Valiyaveetil, S., Go, M.L., Manjunatha Kini, R., Velazquez-Campoy, A., Sivaraman, J., 2008. Structural characterization of myotoxic Ecarpholin S from *Echis carinatus* venom. *Biophys. J.* 95, 3366–3380.
- Zhu, J.-y., Ye, G.-y., Hu, C., 2008. Molecular cloning and characterization of acid phosphatase in venom of the endoparasitoid wasp *Pteromalus puparum* (Hymenoptera: Pteromalidae). *Toxicon* 51, 1391–1399.