Non-invasive extraction of *Cnidarian* venom through the use of autotomised tentacles

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Summary

The animals contained within the phylum *Cnidaria* (Sea Anemones, Corals, Sea Pens, Jellyfish, Boxjellies and Hydra) have origins that can be dated back to around 750 million years ago (mya) and as such, they represent what is potentially the oldest known venomous lineage that is recognised today.[1,2] The phylum *Cnidaria*, which includes Sea Anemones, Corals and Jellyfish are also one of the most understudied as far as toxins go, likely a result of the constraints involved in obtaining samples. Over the last two decades there have been increased efforts to further our ability to obtain samples, however, the sampling techniques developed were invasive and generally required the dissection of tissues from the organism. Within recent years, there have been some developments in the chemical extraction of *Cnidarian* venom, using ethanol to trigger nematocyst firing. These developments have led to the formation of this research, which uses ethanol to elicit stimulation of nematocysts on naturally autotomised tentacles whilst being observed under light microscopy, before having protein content measured using micro-spectrophotometry. This paper focusses on a unique observation of *Cnidaria* that is unknown in any other animal taxa, passive autotomy of envenomation apparatus, the tentacles.

Introduction

Cnidaria is an exclusively aquatic phylum of invertebrate animals which have been shown to have diverged between 686-819 mya, which implicates the entire taxon as one of the oldest venomous lineages that are still present to the current day.[1,2,3] Although the taxa are well known as being venomous, with some species being renowned as medically significant to humans, they have been relatively underutilised as research subjects, despite being a promising and almost untapped source of novel proteins.[4,5,6] One of the largest attributes to the underutilisation of *Cnidarians* in the field of venom research stems from the difficulties encountered when attempting to obtain samples of venom.[4,6]

Since the 1960s the ability to obtain samples of venom and develop our comprehension of *Cnidarian* toxins has gradually increased.[7] However, most of the sampling techniques that have been utilised have commonly necessitated dissection of the organism's tissue to acquire toxins. There have been diverse methods of extraction employed over time, using procedures such as; triggering the release of nematocyst with amnion obtained from human embryos; chemical or osmotic discharge; using mechanical means to achieve the destruction of tissues; sonication of tissues to rupture cnidocytes; maceration and using bead mills to homogenise tissues; however, it has been stated that many of these methods are either of unknown reliability or can lead to proteins contaminating the samples.[6,7,8,9,10,11]

Over the last 5 years there has been progress made in the use of chemical induced firing in order to sample *Cnidarian* venom. A significant conclusion from this research highlights that ethanol is an effective means to chemically initiate the firing of nematocysts, with a secondary advantage being that this method significantly mitigates the contamination of the samples with lysed tissues and non-target proteins.[6,7,8]

Cnidaria, like many other invertebrate taxa, are capable of autotomy of tissue, these mechanisms are presumed to have evolved to protect the rest of the organism.[12] Long trailing tentacles evolved for prey capture can inadvertently entangle other jellyfish or excessively large prey and predators in which it may be advantageous to autotomise the entangled tentacle to

escape. As in other autotomising organisms *Cnidaria* have co-evolved a regenerative ability to maintain function. Casual observation of medusa stages in captivity has highlighted that autotomy frequently happens, potentially through contact or entanglement with tank mates. Authors have also anecdotally observed and identified autotomised tentacles in the coral sea of Australia, highlighting that there may potentially be some slight *in situ* implications for aspects of this study.

As our current understanding of *Cnidarian* toxins is comparatively lacking when considering the research outputs focussing on terrestrial organisms within the field of toxinology,[6,13] a focus on developing further knowledge of the toxins that are produced within this taxa will not only increase our comprehension for their medical significance but will also generate further research of the potential for *Cnidarian* venom based novel drug leads. As the advancements of new extraction techniques have provided a multitude of opportunities, the work presented within this paper aims to provide a novel means of obtaining *Cnidarian* toxins whilst simultaneously improving their welfare. As a result of this, there may be potential to increase our current knowledge base of *Cnidarian* venoms whilst promoting a non-invasive means of sample acquisition.

Ethical considerations

The *Cnidarian* used within this research are not protected under the Animals (Scientific Procedures) Act 1986. However, prior to undertaking any research, all aspects of the *Cnidarian* work detailed within this paper were subjected to an ethical application and subsequent ethics review by the ethics committee at The Deep Aquarium (The Deep), Hull. Furthermore, the research has been performed with the improvement of ethics in mind and as such has been carried out in a manner that promotes zero live animal contact through the use of tissues obtained as a result of natural autotomy. Rat erythrocytes were harvested in accordance with the 3Rs and Animals (Scientific Procedures) Act 1986 from Schedule I culled animals used for other studies.

Methods

Husbandry

The *Chrysaora* medusae (adult life stage) are kept and displayed in a kreisel system (Figures 1 and 2), a specially designed aquarium specifically for housing pelagic jellyfish and Ctenophores.

The jellyfish display system consists of a large kreisel with a volume of 824L, a smaller kreisel with a volume of 297L, a sump containing around 1,000L and altogether including pipework and life support the system (Figure 3) holds a total volume of 2,222L of artificial saltwater.

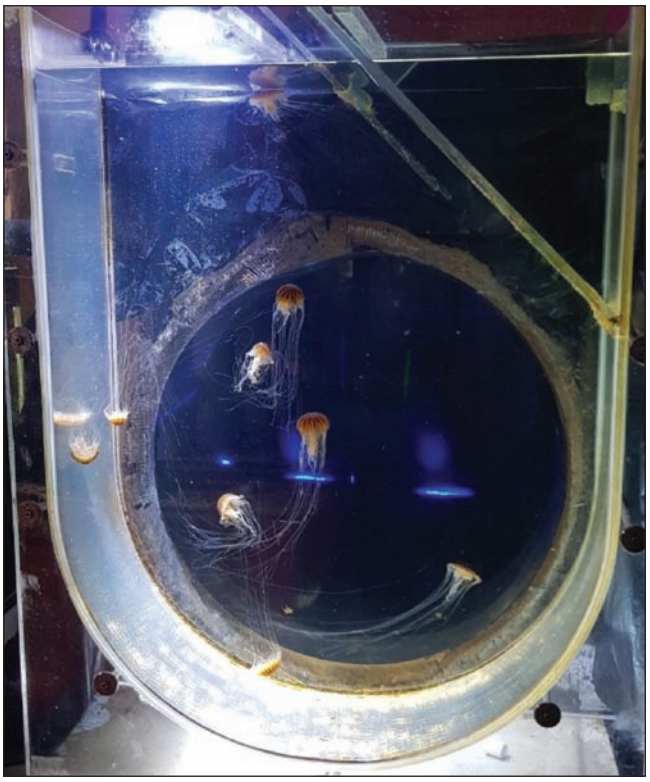

Figure 1. A 274L kreisel system housing *Chrysaora pacifica*. This image, taken from the back of the system with the backdrop removed, illustrates the general set up and plumbing of a kreisel system. It is worth noting that this specific enclosure is technically a pseudo-kreisel as a true kreisel is fully rounded rather than possessing a flattened wall as in this image.

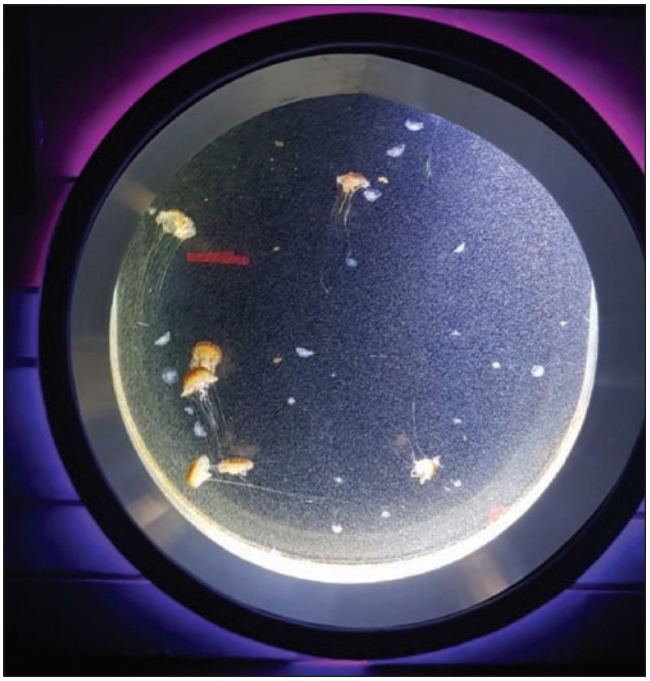

Figure 2. The same kreisel system depicted in Figure 1, this time from the exhibition side with the backdrop in place. Note that the jellyfish have just been fed and small *Aurelia* can be seen as well as a cloud of *Artemia* nauplii.

This is made on site at The Deep using 1 tonne of Bromine free aquarium salt (Aquarium Solutions, France) dissolved in 33,000L of reverse osmosis (RO) water before being pumped around the building for use in all marine systems. The circulation of the water inside the kreisel flows in a circular, gyre-like current, which ensures that the jellyfish remain constantly suspended within the water column and do not suffer tissue damage due to constant contact with hard surfaces of the system and plumbing. System water drains through an overflow box and runs to a sump containing the life support system, which includes; a sand filter (Waterco T500); a trickle tower biological filter (TMC TBT5300P); a protein skimmer (TMC PSW5200P); glycogen fed chiller plates; and a 4 tube UV sterilisation and clarification system (TMC P4 220W).

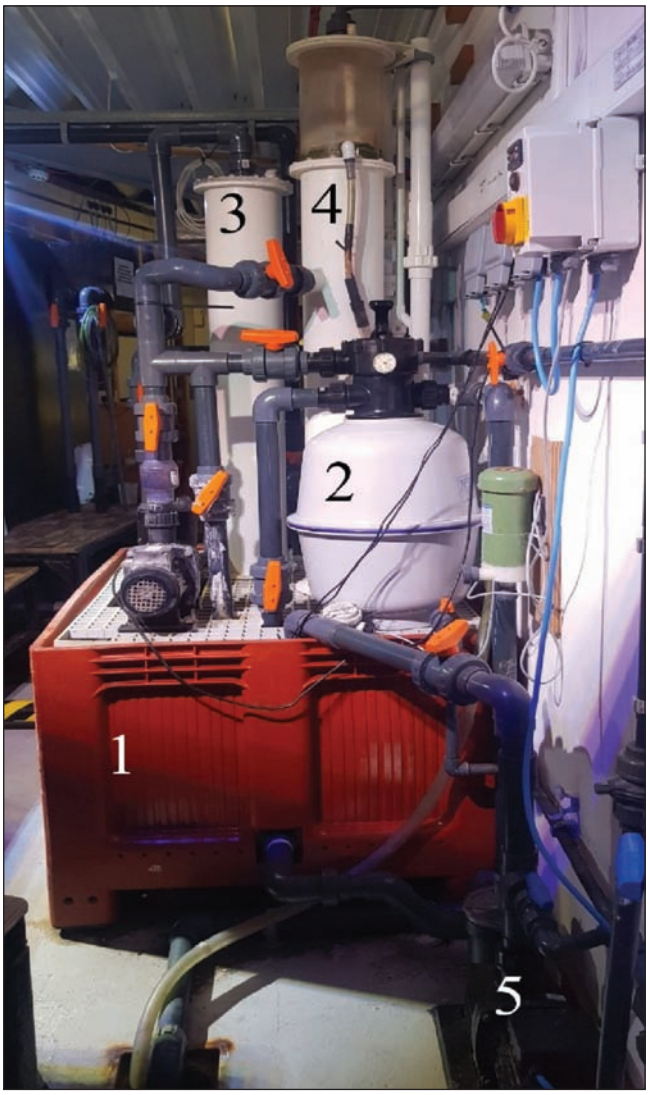

Figure 3. A view of the LSS for the *Chrysaora* spp. kreisel systems. Within this image you can see; 1 – sump; 2 – sand filter; 3 – biological trickle tower; 4 – protein skimmer, a rapidly aerated body of water in which foam fractionation occurs, removing proteinaceous waste from the water; 5 – circulation pump that feeds the chiller loop.

The water quality parameters are maintained as follows; salinity – 33ppt; pH – 8.1; Ammonia (NH_3) – 0mg/L; Nitrite (NO_2) – 0mg/L; Nitrate (NO_3) – <10mg/L. Water changes of around 25% are performed 3 times weekly, this occurs regardless of whether water quality is within the specified parameters or not, due to anecdotal evidence of a decline in the body condition of the jellyfish through periods without water change. Multiple smaller water changes are favoured over fewer large changes as it reduces the severity of fluctuations that occur in water quality as a result. Routine maintenance includes keeping surfaces clear of algae, debris and hydroids through manual removal with sponges and siphoning. Alongside this, there is also the occasional requirement to remove the animals from a system and perform a thorough disinfection of the equipment. This, however, is only generally undertaken when hydroid levels become significant as transfer and handling of jellyfish can be stressful for the animal. Should a thorough clean be required, the individual kreisel is initially isolated from the system before a 5% sodium hypochlorite solution (Total Pool Chemicals, Cheshire, UK) is added to a concentration of 0.61% and then left in the kreisel for a period of 2-3 hours. It is then drained of all saltwater, rinsed and filled with fresh tap water and the sodium hypochlorite is neutralised through the addition of sodium thiosulphate ($Na_2S_2O_3$) (Acros Organics, Belgium) at a rate of 6.99g/L of sodium hypochlorite present in the system. As the hypochlorite is reduced by the thiosulphate there will be a slightly yellow appearance to the system water, before it gradually becomes clear again and the chlorine-like aroma dissipates. At this point, the system is drained and thoroughly rinsed with fresh tap water to remove any traces of chemicals, before being drained and finally refilled with artificial sea water.

The scyphistoma (polyp) life stage of *Chrysaora* are housed in a 3L Really Useful Box® (Really Useful Products©, Castleford, UK), which is partially submerged within a large water bath with several other tubs containing polyps (Figure 4). The water is maintained at 17-20˚C within the bath using 150W heaters (Aqua-El Ultra heater) and a chiller unit (Aqua

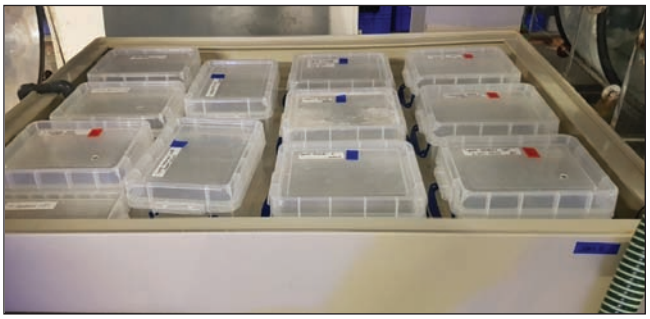

Figure 4. A water bath containing multiple 3L Really Useful Boxes housing a number of polyps, each containing a different species of *Chrysaora* spp.

Medic Titan 2000) to ensure temperatures do not unintentionally fall outside of these parameters.

A 90-100% water change is performed on the tubs every two weeks on average; however, the frequency in which this may be required can often depend on the number of polyps present and factors such as algae build up. To induce strobilation (metamorphosis) from the polyp stage into the ephyra stage, the temperature of the water bath is very gradually increased by 1-2˚C at the rate of around 1˚C per week. The water is then held at this temperature for around a week before being slowly reduced by 3-4˚C over the space of at least a week. The strobilation is induced by the stress of enduring the changing temperature and once the cue to strobilate has been provided, it may take a minimum of 2-4 weeks before any ephyrae are produced, depending on the species in question. Before strobilation is initiated the polyps must be in good condition, which can be ascertained through having a plump appearance with an almost pink colouration to them. As strobilation occurs the polyps will be seen to elongate and the colour will change to brown. At this point the ephyra will begin to detach from the strobilae and become free swimming individuals.

Ephyrae can be carefully transferred from the tub into a 5L glass fishbowl that is gently aerated through a 6mm airline attached to a diaphragm air pump (Hiblow® HP-40); the aeration should be gentle enough to ensure ephyrae remain suspended in the water column but not so severe that they are unable to freely swim through pulsating of the bell. Transfers of ephyrae should be performed using a plastic Pasteur pipette, which will need the tip cutting so the hole is larger than the size of the ephyra. If the ephyra is too large to fit in a pipette, the transfer should be performed using a plastic or glass beaker. As each ephyra grows and their lappets begin to fuse into a bell, they require carefully transferring from the glass fishbowl into a small 30L kreisel. Initial flow within the kreisel should be much lower than is required in a kreisel housing fully developed medusae but this will necessitate gradual increases as the ephyrae develops in size and becomes a medusa. When increasing the flow of the kreisel it is imperative to ensure that the increase is done over several days or more as to minimise stress, potential tissue damage and the possibility of tentacles becoming tangled, once they have developed.

Unlike some species of jellyfish, *Chrysaora* do not have a symbiotic relationship with zooxanthellae and as such are not photosynthetic, which means that each life stage requires active feeding. The type of food and frequency of feeding changes as polyps grow and metamorphose, with polyps requiring feeds at least 5 times a week using between 1-3mL of either live rotifers (L-strain (*Brachionus plicatilis*) (ZM Systems, UK)) or live nauplii. When using live rotifers the cultures, which are kept in a lower salinity of 20-22ppt, should gradually be brought up to the same salinity of the system that is being fed. Live rotifers are the best staple to provide when feeding the ephyra life stage, although supplementary power feeding several times a week is often the most ideal method to ensure that enough food is acquired. This is undertaken using around 150mL of system water in a beaker and adding 1-2mL of live rotifers or live nauplii and allowing the ephyrae to feed for around 10 minutes, keeping a close eye on water quality during this period. As the ephyrae grow and start to form a bell, additional power feeds of mucous taken from *Aurelia* species are provided several times a week for a duration of no longer than 10 minutes. This gradually increases into being target fed small chunks of chopped *Aurelia* as the tentacles develop. Adult medusae are fed 3 times a day with the first being a targeted feed of roughly chopped *Aurelia* chunks which are squirted into the tentacles and mesenteric arms of each individual using a turkey baster, this is supplemented with 800-1200mL of live nauplii (dependent on the size of the kreisel, stocking density and the volume of *Artemia* in the culture). The second feed that is offered is a 'free feed' of around 800mL live nauplii suspended in the water column and the final feed of the day is either defrosted *Calanus* species or occasionally defrosted *Mysis* species along with another 800-1200mL of nauplii. *Calanus* species is the preferred food as it remains suspended in the water column for a greater length of time, being a smaller organism. The nauplii that are used are cultured on site and are always decapsulated using sodium hydrochloride before culturing takes place.

Decapsulation protocol

The *Artemia* cysts were rehydrated in fresh water that was aerated using an air pump and air stone for approximately 2 hours. All the water from the rehydrated *Artemia* cysts was strained away using a fine filter sock. 70g of sodium hydroxide was added to a bucket containing 2.5L of fresh water and allow to dissolve. In a separate container, 1.65L of sodium hypochlorite was added to 3.35L of fresh water and then mixed. This was then added to the sodium hydroxide solution. The filter sock containing the *Artemia* cysts was then added into the mixed solution, ensuring that the top of the sock remained out of the water, so cysts were retained within the filter. As the reaction took place, over several minutes and caused the decapsulation to occur, the cysts changed colour from their original brown into an orange colour. Whilst the decapsulation was taking place, a solution of sodium thiosulphate was made in a separate bucket by dissolving 70g of sodium thiosulphate in 6L of fresh water. Once around 90% of the cysts appeared to have changed, the reaction was inhibited by removing the filter sock and rinsing with fresh water thoroughly before the filter sock was placed into the sodium thiosulphate

solution and soaked for around 2-3 minutes. After the filter sock was removed from the sodium thiosulphate solution it was rinsed under fresh water for several minutes before having all the excess water strained away. Decapsulated cysts were stored in a fridge, inside a container with just enough artificial saltwater to cover the cysts. To hatch the *Artemia*, the desired volume of cysts was placed into a vessel containing artificial seawater that was heated to 26-28˚C and aerated for around 24 hours. Ensuring that the aeration was strong enough to ensure the cysts remained suspended within the water column throughout.

Venom extraction procedure

In order to conduct the primary trials for this method, a small section of naturally autotomised tentacle was removed from the kreisel using a 30mL Universal specimen bottle and was stored in system water at 4°C until it was processed later the same day. This was to initially assess whether the nematocyst firing would be elicited using this method. The small section of tentacle (~2mm) was placed onto a clear glass microscope slide and as much of the remaining seawater as possible was blotted dry before the slide was placed onto the stage of the microscope (Olympus BX40). Once the view had been focussed at a magnification of x20, a small amount of ethanol was added to the slide using a Pasteur pipette to initiate the firing of the nematocysts.

The secondary stage of development was to assess the extract for the presence of protein and, in turn, ascertain if there was the potential presence of a usable amount of venom within the sample. This was achieved through placing a section of tentacle (~10mm) into a 0.5mL centrifuge tube containing ethanol and analysing the quantity of protein found within the solution using a DS-11 Spectrophotometer (Denovix®, USA). After blanking the microvolume reader, a 1µL sample of the ethanol/tentacle extract mix was pipetted onto the reader and the protein concentration was measured using absorbance at 260/280nm, using the pre-set BSA calibration. This process was repeated at 1-minute long intervals in order to calculate the ideal immersion time required to obtain the largest possible extract volume within the sample (Figure 5).

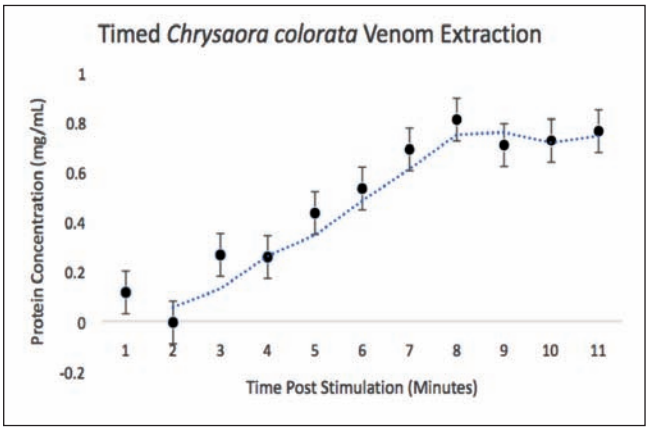

Figure 5. Rate of protein release into extraction media from autotomised tentacle.

Once a guideline for timings had been estimated, a new sample was produced; this time using two sections of tentacle (one measuring ~110mm and the other ~72mm). The tentacles were immersed in ethanol for around 8 minutes before being removed to prevent contamination of the sample through tissue lysis.

Sample preparation

Samples were concentrated using 0.5mL 10,000 Da centrifugal filters (Amicon® Ultrafree®-MC) and each 10K filter was centrifuged (Hermle Z160M) at 5000g for 10 minutes. The retentate from within each filter tube was then pooled into a separate 0.5mL centrifuge tube. Owing to the potential instability of *Cnidarian* toxins,[14,15,16] the extracts were lyophilised to improve stability during transport of samples from the sampling site to the laboratory. The lyophilisation was achieved using a desiccation method; attained by placing the centrifuge tube containing the sample into a 530mL airtight food-grade lockable container (Sistema® To Go™, Sistema® Plastics UK Limited, Surrey, UK) containing 50g of silica gel SiO_2, with the lid of the centrifuge tube left open. The container was then sealed shut with Parafilm® wrapped around the lid to ensure the seal was as airtight as possible. The sample was desiccated at ambient temperature ~26˚C until it had been fully lyophilised, which took around two days for an entire 0.5mL tube. The lyophilised samples were then stored at -20˚C as an additional measure to preserve the integrity of the extract.

Protein analysis

The lyophilised venom was dissolved in 40ul of HPLC grade water. The DS-11 spectrophotometer was blanked with 1µL HPLC grade water, wiped and the three successive 1µL aliquots of extract were measured for protein concentration.

Erythrocyte lysis

Rat erythrocytes were collected passively into heparinised phosphate buffered saline (PBS) (Scientific Lab Supplies) post cervical dislocation and decapitation. Cells were chilled on ice until counted with a haemocytometer. In a conical well microamp 96-well plate (Applied Biosystems) 100µL of 10mg/ml venom along with separate rows of 100% Dimethylsulphoxide (DMSO) (Fisher Scientific) and 100uL sterile water was serially diluted 1 in 10 into PBS. 100µL of $1x10^7$ cells/ml was added to each well and the plate covered with sealing film and incubated at 37°C for six hours. Supernatants were removed from each well into a polystyrene 384 well plate and read on a Fluostar plate reader (BMG Labtech) at 414nm and 620nm as per Eschbach *et al.*[17]

Results

Light microscopy of nematocysts firing

Nematocysts were observed to fire from the tentacle in response to ethanol stimulation. Most nematocysts were fired within the first 12 seconds, with a relatively small number being slower and a complete halt in firing being observed from around 20 seconds. Figure 6 shows tentacle section before stimulation, Figure 7 shows tentacle post firing of nematocysts (bright white lines protruding from tissue).

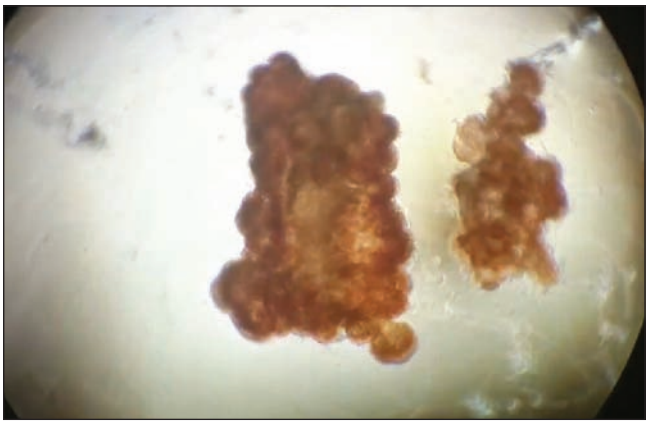

Figure 6. A small section of autotomised tentacle obtained from *Chrysaora pacifica*, observed Pre-firing at x20 magnification.

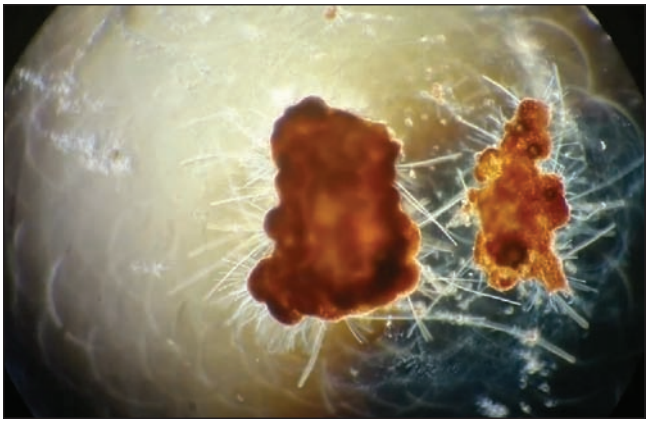

Figure 7. A post-firing image of the tentacles seen in Figure 5. The fired nematocysts can be clearly observed as white lines extending from the segments of tentacle.

Protein analysis

The investigation of rate of protein (venom) release into stimulation media (Figure 5) demonstrates that although the nematocysts have finished firing after 20 seconds the protein continues to increase before reaching a plateau at eight minutes. This could be because the venom is either being released for longer or it is an artefact of diffusion of the released venom into the stimulation media.

The 40µL sample stimulated in microcentrifuge tube contained 89.57 (+/- 3.6) µg/µL protein, thus 3.583mg of protein was obtained. The high ratio of 260/280nm 0.94 (+/-0.01) indicates nucleic acid contamination, presumably from cellular lysis from the broken ends of the tentacle but which may also be a component of the venom itself.

Identification of active venom

At a final concentration of 5 µg/µL *Chrysaora* venom from autotomised tentacles caused a modest amount of lysis in rat erythrocytes (Figure 8). This caused release of 13.3% of haemoglobin into the supernatant when compared to the positive control of 50% DMSO. Although small, the effect is significantly different ($p=0.013$) from the negative control (PBS) i.e. null hypothesis of no effect, using a non-paired student T Test in Excel (Microsoft).

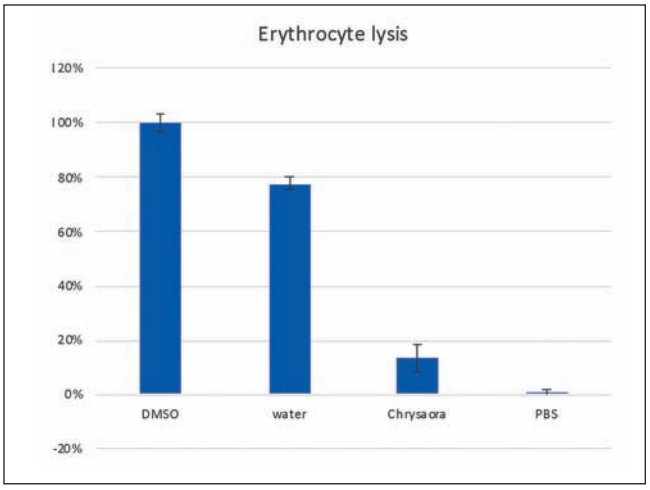

Figure 8. Erythrocyte lysis of *Chrysaora colorata* venom compared with 50% DMSO (control) and 50% sterile water. The venom collected from autotomised *C. colorata* tentacle causes 13.3% (+/- 5%) lysis of isolated rat erythrocytes.

Discussion

This study demonstrates that protein concentration increases in the media surrounding fired nematocysts from autotomised tentacles. This protein has venom like properties of causing erythrocyte lysis and thus is expected to be venom that would have been injected by the animal on envenomation. Further work is planned to characterise the venom such as identifying proteins contained within and their functional effects. Thus, further development could lead to a novel means of obtaining *Cnidarian* venom samples without the need to undertake any animal contact. Despite the potential beneficial implications linked to this study, there are also some areas in which these techniques are less efficient than the currently accepted methods. Some of the foreseen downfalls associated to the use of the autotomy method are; the unreliability of sample availability; the inconsistent size and weight of tentacles that can be obtained; and the potential loss

of tissue integrity encountered from autotomised tentacles going unnoticed for a length of time. Further work is expected to include investigation of autotomised tentacle longevity in order to examine how long they retain viability post-autotomy; refining the processing and storage of samples; and developing a standard for sample quality which allows for the most efficient assay results.

The results indicate nucleic acids are present in the venom this could either be from the broken ends of the tentacles or the venom itself. Nuclei acids such as RNA and DNA have been reported from vertebrate venoms so may be a part of the normal composition of Cnidarian venoms. [19]

This study highlights an unusual aspect of *Cnidarian* biology that presents a unique opportunity to collect and study the venom of these animals without trauma or euthanasia, and thus we feel is a major improvement in their welfare.

Acknowledgements

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