Research Article

Comparative evaluation of polyclonal antibodies in the characterization of nematocyst proteins from Australian Irukandji and *Chironex fleckeri* species

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Abstract: *Carukia barnesi* (*Cb*), *Malo kingi* (*Mk*) and *Chironex fleckeri* (*Cf*) are dangerous Australian box jellyfish species that provoke distinct and not well understood envenomation syndromes. Specifically, *Cb* and *Mk* are small, rare and able to induce a systemic syndrome of generalised muscle pain and catecholamine excess termed "Irukandji syndrome"; *Cf* has been widely regarded as one of the most venomous organisms in the animal kingdom causing severe sting site pain combined with potentially lethal cardiotoxicity. Building on past studies of major chirodropid and carybdeid species venoms, this study compared the utility of various cubozoan specific antibody reagents to better define the relationships between venom proteins from both exemplar Irukandji species (*Cb* and *Mk*) and the archetype *C. fleckeri* box jellyfish. With the aid of commercial ovine derived *Cf*-specific antivenom, mouse antibodies reactive to *Cb* and *Mk* and rabbit antibodies specific to two *Cf* toxins (CfTX-1 and 2), as well as human sera, the cross-reactivity of jellyfish species-specific polyclonal antibodies against these three cubozoan venoms was investigated. Immunoblot assays revealed distinc levels of immune recognition across the three species, indicating that *Mk* specific reagents may bind both Irukandji and *Cf* venoms. Irukandji venom appears to be antigenic with the exception of a few proteins in the range of 43/46 kDa maybe homologous to CfTX-1 and 2. The implications of such antibody binding for future antivenom development require further investigation.

Keywords: Irukandji syndrome, box jellyfish, CSL antivenom, nematocyst extracts, antigenicity; human sera, human antibodies.

1. Introduction

The class Cubozoa ['box jellyfish'] within the phylum Cnidaria consists of two orders: the Carybdeida and the Chirodropida (**Figure S1**). Members of this phylum are characterised by the presence of unique cells stinging cells (cnidae) [1] that are controlled by the nervous system; the commonest of these are known as nematocysts. These are sophisticated "single-use" mechanical sensor capsule-like attack devices [2], that comes in a wide variety of sizes and shapes, and typically evert a barbed harpoon. The lumen of the capsule contains a complex arsenal of peptides, polypeptides and bioactive lipids directed to trap, immobilize and ultimately kill prey [3]. Nematocysts in Cubozoa are arranged in rings along the tentacles, and, in some species within the Irukandji group, may also contain random colored warts on the exumbrella that present clusters of a different type of nematocyst. The Irukandji group consists of at least 16 species ranging in size from diminutive to very large [4]. The sting of these jellyfish manifests on a spectrum from mild to potentially lethal depending on the particular stinging species. From smallest to largest, Irukandji jellyfish include: *Carukia barnesi* [6], *Carukia shinju* [7], *Malo bella* [9], *Malo kingi* [8], *Malo maxima* [7], *Gerongia rifkinae* [10], *Alatina mordens* [7], *Morbakka fenneri* [5], *Keesingia gigas* [9], and others.

The Irukandji syndrome is named after an aboriginal tribe that inhabited the Palm Cove region in North Queensland, Australia, where a marine envenomation of initially unknown aetiology was first reported [11,12]. The envenomation was formally named and publish by Dr Hugo Flecker in 1952 as occurring around Cairns, North Queensland, with *C. barnesi* [13] (*Cb*) being subsequently identified as the primary causative agent for cases in that region [11,14]. The syndrome caused envenomation by such species is a collection of signs and symptoms of variable severity, beginning with slight sting site pain, reddening of the skin followed by a delay of ~30 minutes before the onset of potentially severe systemic features, these include severe lower back pain, nausea, vomiting, difficulty breathing, generalised sweating, panic/anxiety attacks, muscular spasms, abdominal cramping and elevated blood pressure. In some cases cardiopulmonary decompensation leading to pulmonary eodeme has been reported [4]. Affected individuals may have a combination of these features, but consistently suffer musculoskeletal pain often focused around the trunk [15]. In more severe cases intracerebral hemorrhage has been observed after severe systemic hypertension, causing death [16], thought to be secondary to catecholamine excess. Two fatalities from Irukandji syndrome were reported in 2002: one in the Whitsunday Islands, and the second off Port Douglas in the tropical Great Barrier Reef [17]. According to some authors [18,19], the nematocysts obtained from one of the 2002 victims were identified as a unique nematocyst type implicating *M. kingi* (*Mk)* as the most likely cause of death [19]. Further, in May 2013, the deaths of two people snorkelling off Coral Bay, Western Australia were potentially caused by an Irukandji jellyfish [20]. Irukandji envenomations are not limited to North Queensland [14] as cases exhibiting the characteristic syndrome have been reported in Broome on the West Australian coast [21,22] and as far south as Victoria [23]. Irukandji syndrome is also not restricted to Australian waters as cases of the syndrome are documented throughout tropical and temperate oceanic regions of the world [4,24], such as the reefs off south Florida [25], the French West Indies in the Caribbean [26], Thailand [27, 28], Malaysia [29] Hawaii [30], East Timor and Papua New Guinea [31].

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The difficulty of studying the venom of Irukandji syndrome-causing jellyfish is compounded by a number of factors. Besides being hazardous to collect, these species are rare, seasonal, often small and colourless, with minimally characterized lifecycles. Consequently, since the collection of the first causative species in 1961, venom analysis, and hence the development of a specific treatment, has been quite slow [4]. Nevertheless recent studies have provided valuable insights of the biological activity and action mechanism in *Cb* venom. For instance, the effect of whole *Cb* venom on the cardiovascular tissue from humans, rats and guinea-pig (*in vitro)* and on anaesthetized piglets (*in vivo)* has been reported [6], Whole *Cb* venom caused tachycardia and systemic pulmonary hypertension triggered by a massive release of neurotransmitters, notably the catecholamines adrenaline and noradrenaline [6]. These initial results have, in part, been supported by a second study regarding the *in vivo* effects of nematocyst proteins from *Cb* as well as tentacle extracts devoid of nematocysts tested in anaesthetized rats. The difference between the two extracts in the second study was that nematocyst proteins produced cardiovascular collapse whereas the tentacle tissue extract did not [32]. The experimental results of both research groups and others [33] are consistent with features of catecholamine excess typical of the clinical syndrome [34]. Although these experimental results explain some of the features observed in Irukandji envenomation, the component responsible for the stimulation of excessive sympathetic neurotransmitter release has never been purified nor has any other *Cb* derived toxin-related protein.

In contrast, currently the best characterized cubozoan venom, in terms of transcriptome and proteome expression, as well as toxin functional characterization, is that of the multitentacled *Chironex fleckeri* (*Cf*) box jellyfish [35]. Historically this species has been responsible for the most severe jellyfish envenomations, and, although most are not life threatening [36], by 2004 more than 78 people have died from *Cf* stings [37-40] [41] [20,42]. *Chironex fleckeri* occurs in high abundance [43], is of large size and has numerous tentacles with millions of tentacular nematocysts that contain potent, fast-acting toxins. The most abundant jellyfish toxins belong to the pore-forming toxin family identified in *C. fleckeri* CfTXs [35], *Chironex yamaguchii* (as *Chiropsalmus quadrigatus)* CqTX-A [44], *Alatina sp* CaTX-A, *Carybdea brevipedalia* (as *Carybdea rastoni)* CrTXs [44], *Malo kingi* MkTXs [45], in other cnidarians such as schyphozoans [46], hydrozoans *e.g Hydra magnipapillata*, *Hydractinia symbiolongicarpus* and *Hydra vulgaris* [47,48] and even in the Anthozoa, the basal Cnidaria class *e.g Aiptasia pallida*. Members of the pore-forming protein family have been proven experimentally to play a pivotal role in cubozoan envenomation [35]. Considering the evolutionary position of Cnidaria in the tree of life, this pore-forming toxin family may be well considered a cnidarian innovation [48].

Box jellyfish antivenom prepared by the Commonwealth Serum Laboratories (CSL AV).

Since 1970 the only commercially available jellyfish antivenom has been that produced in Melbourne, Australia, by the company now known as Seqirus (formerly the Commonwealth Serum Laboratories [49]). This product is the result of hyper-immunizing sheep with venom obtained by the electrical [50] stimulation of *Cf* tentacles. Although CSL antivenom has been available for more than 30 years, its effectiveness in treating human envenomations has been contested [49]. Indeed three casualties have occurred despite administration of the antivenom [51-53]. Nevertheless *Cf* specific antivenom is demonstrably effective in attenuating experimental venom extract induced cardiovascular toxicity [54] and continues to be recommended for severe envenomations. Indeed, experiments in animal models (piglets and rats) have shown that the effectiveness of box jellyfish antivenom to counteract lethal, haemolytic, dermo-necrotic and pain-inducing effects is markedly reduced when administered against crude extracts that were obtained by mechanical disruption of nematocysts, in comparison with milked venom and whole tentacle crude extracts [55,56]. Thus, CSL antivenom may possibly lack high affinity antibodies against some venom constituents [57]. This may reflect the presence of non-toxin antigens from jellyfish carbohydrates and lipids, as well as

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proteins from nematocyst capsules and tentacle structural tissue [58]. It is certainly the case that this CSL antivenom does not neutralize Irukandji toxins to any clinically significant degree [6,49,50,59,60]. Hence, the lack of broadly reacting high affinity cubozoan venom neutralising therapies remains an ongoing challenge to the specific management of both Irukandji and *Chironex-type* envenomations.

Cnidarian toxins often contain similar primary and/or secondary structural motifs [47,61,62] reflecting potentially similar function, as well as antigenicity, to their homologs in related species [63]. Logically, the phylogenetic relationships between chirodropids and carybdeids suggest that antibody-binding regions within the toxins of each species group might be explored to identify homologous toxins across this cnidarian class. Building on our past studies of major chirodropid and carybdeid species venoms [6,50,54,64,65], this study compared the utility of various cubozoan specific antibody reagents to better define the relationships between venom proteins from both exemplar Irukandji species (*Cb* and *Mk*) and the archetype box jellyfish (*Cf*). Specifically murine antibodies raised against native *Cb* and *Mk* venom, antibodies specific for a 42-46 cytolytic protein doublet of *Cf,* the CSL polyvalent ovine whole *Cf* venom specific antivenom, and sera from cubozoan jellyfish sting sensitized humans, were compared for binding to selected whole nematocyst venoms across this cnidarian class.

2. Results

2.1. Nematocyst extract preparations

Carukia barnesi venom proteins were studied using three different venom extracts. The first bell crude extract (BCE) corresponded to proteins isolated from spherical anisorhiza nematocysts located in red wart-like clusters at the umbrella (**Figure 1 A and E**) and the second sample: tentacle crude extract (TCE) was prepared from microbasic mastigophore nematocyst present in the tentacles (**Figure 1 B, C and E**). These two preparations derived from five pooled and dissected juvenile medusae (See methods section) obtaining ~12 and 18 µg of total protein, respectively. A third kind of sample was prepared from nematocysts from both the umbrella and the tentacles, using adults or mixed stages (mostly juveniles) of *Cb* with a typical total protein yield of 30 to 38 µg; these preparations are referred to as whole crude extract (WCE). In contrast, *Mk* WCE was prepared only once using nematocysts from both the umbrella and the tentacles of a single adult medusa with a yield of 25.35 µg of total protein. *Cf* WCE preparation yielded total protein of ~100-200 µg depending on the nematocyst preparation.

The same protein batch of WCE of *Cb* and *Mk* respectively were used for mouse immunization and dot blots (see below).

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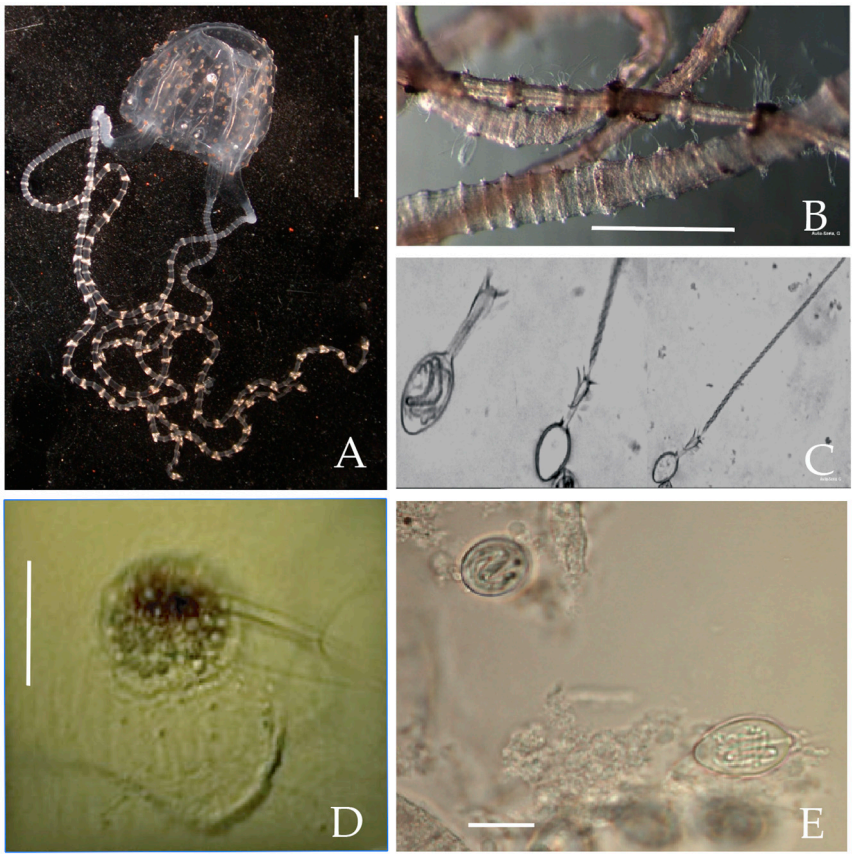

Figure 1 Imaging of whole *Carukia barnesi* jellyfish, tentacles and isolated nematocysts. A, Mature whole specimen; B, close up of elongated tentacles with a tailed pattern and attached fired nematocysts; C, firing of a nematocyst; D, reddish bell wart with clusters of spherical anisorhizas, some of them fired; E, spherical anisorhizas (on top left) and microbasic mastigophores -tentacular mastigophore nematocysts- (lower right). Scale bars in A = 3 cm; in B = 0.15 cm and in D and E, = 20 μm. Photographs A and E copyright L. Gershwin.

A lack of *Mk* material prevented the elucidation of *Mk* venom profiles, however, the WCE prepared from both types of spherical bell isorhizas (**Figure 2 C**) and tentacular microbasic mastigophore nematocysts from *Mk*, as well as from *Cb*, were used to raise antibodies in mice and, importantly, to assess the specific and overall antigenicity of the venom proteins in the dot blot assay (see below). Samples of *Cf* nematocysts were of crucial importance as they were consistently more available and had nematocyst extracts with higher concentrations of venom components, when compared to those obtained from Irukandji species, and consequently, *Cf* extracts were preferentially used to develop the study protocols, and as positive controls in both venom profiling and cross reactivity studies.

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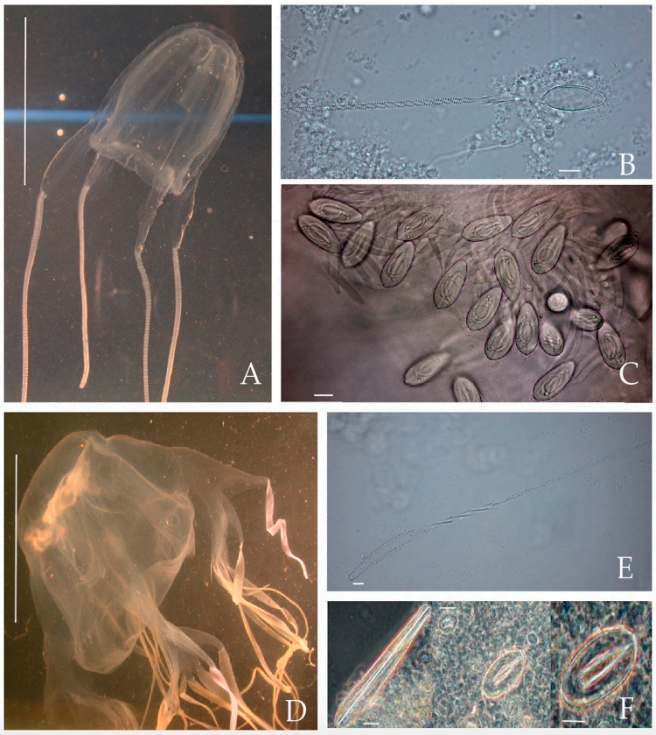

Figure 2 Imaging of whole *Malo kingi* and *Chironex fleckeri*. A, a mature specimen of *Malo kingi*; B, discharged tentacular microbasic mastigophore nematocyst showing the fired strand and its spines full length; C, arrangement of tentacular unfired microbasic mastigophore nematocyst from *Malo maxima,* a close relative to *Malo kingi* with similar nematocyst features; D, a mature *Chironex fleckeri*; note the multi-tentacled fettuccine-like tentacles; E, type 3 banana-form large microbasic mastigophores (~ 90 µm in lengh) of *C. fleckeri*. F, undischarged *C. fleckeri* large mastigophore, small trirhopaloid and large trirhopaloid nematocysts (~ 35 µm lengh in average) from *C. fleckeri.* Scale bars in A = 3 cm; in B, C and E = 10 µm; in D = 30 cm, F=10 µm. Photographs A-E copyright L. Gershwin.

2.2. Venom protein profiles

Venom profiles of the three types of extracts from *Cb* are shown in Figure 3. Approximately 4 µg of tentacle nematocyst proteins from juvenile medusae (**Figure 3, Lane A**) were resolved in an apparent molecular range from 19 to 120 kDa. Molecular mass estimations and imaging analysis confirmed the presence well-resolved proteins with major bands corresponding to 36, 43, 45 and 47 kDa, followed by minor bands at, 28, 60, 90 and 120 kDa. Less than 4 µg of total protein prepared with bell nematocyst from juvenile medusae (**Figure 3, lane B**) revealed protein bands between 28 and 120 kDa, with major bands at 28, 42, 47 and the region of 76-78 kDa. Separation of the proteins from *Cb* whole venom from adult specimens (**Figure 3, Lane C**) detected polypeptides ranging in size from 5 to 250 kDa, a complex profile clearly dominated by a tight protein doublet with a molecular mass of about 46 kDa that appeared as a single band but resolved into two bands with the slightly larger band being more predominant. Three µg of a WCE prepared with a batch of mixed stages (mostly juveniles) of *Cb* showed eight resolved bands between 10 to 70 kDa including a doublet in the range of 45-50 kDa (**Figure 3, Lane H**). Typically, *Cf* WCE preparations resolved in profiles similar to **Figure 3, Lane I**, in a range of ~10 to 200 KDa. The profiles were dominated by a broad region between 40-45 KDa followed by other less abundant bands of 150, 80 and 68 KDa and minor abundant bands of 48, 65 to 48 KDa. Bands of less than 28kDa were not consistently observed.

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The denaturing SDS-PAGE profiles indicated differences between the proteins found in the two nematocysts types of *Cb* (**Figure 3, lanes A-B**) and between the WCE of different ontogeny stages from *Cb* and *Cf* (**Figure 3, lane C, H and I**). It is significant that despite heterogeneity and dissimilarity among protein profiles of both species, there are key common proteins, specifically a key doublet around 43-46 kDa that is observed for both *Cb* and *Cf*.

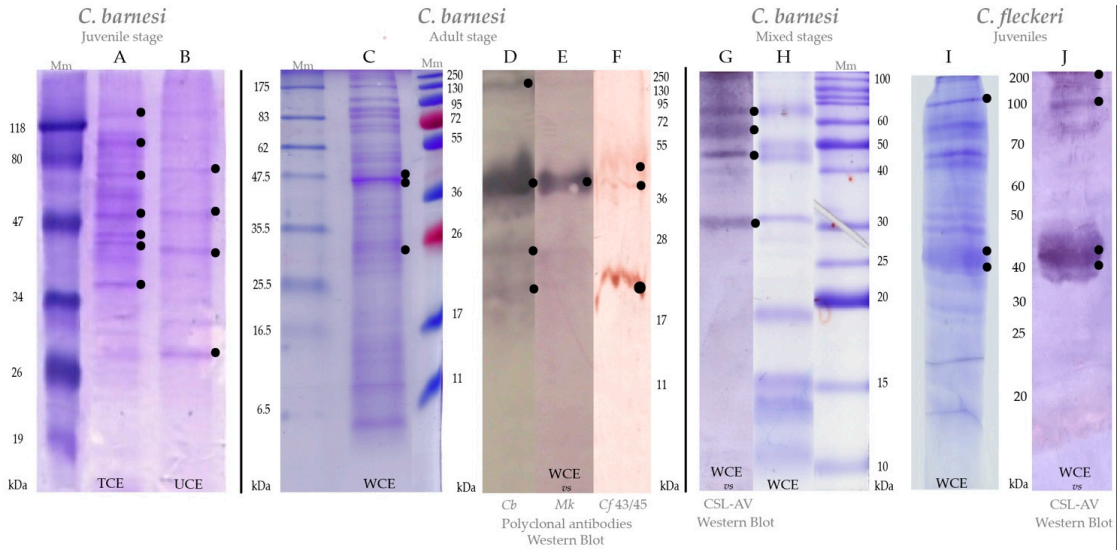

Figure 3 Coomassie blue stained SDS-PAGE 12.5%-15 % (w/v) venom profiles and western blot (WB) analyses of cubozoan nematocyst extracts. In **lane A,** 4 µg of tentacular nematocyst crude extract (TCE) of *Cb*; **lane B**, less than 4 µg of bell nematocyst crude extract (BCE) of *Cb*; **lane C**, extract 5 µg of both types of *Cb* nematocysts (WCE); **lanes D** and **E**, western blot analysis of *Cb* extracts (same protein batch and protein amount as used in lane C), tested for cross reactivity using *Cb* and *Mk* polyclonal antibodies raised in mice, respectively; **lane F**, cross reactivity of *Cb* WCE (same batch and protein amount as used in lane C), against rabbit polyclonal antibodies specific for *C. fleckeri* 43/45 kDa toxins (CfTX-1 and 2); **lane G**, western blot using CSL Cf antivenom against *Cb* nematocyst WCE (different batch than line equal protein amount C); **lane H**, *Cb* nematocyst WCE (same batch and protein concentration [3 µg] compared with lane G). **Lanes I and J**, *Cf* venom profile and western blot towards CSL *Cf* antivenom. Protein standards: NEB, Fermentas SM671 and BenchMark™ (Invitrogen), are shown in kilodaltons (kDa). **SDS-PAGE in lines A-H**, were resolved in 8.3 x 7.3 cm gels of 12.5% (w/v) polyacrylamide. Lanes I and J correspond to 20 µg of total protein, resolved in a 16 x 20 cm gels of 15 % (w/v) polyacrylamide. In the figure, the black dots represent abundant proteins (A-C, J-I) and antigenic protein bands (D-F and J). TCE stands for tentacular crude extract, UCE=Umbrellar crude extract and WCE= Whole crude extract.

2.3. Antigenicity of nematocyst extracts fractionated by SDS-PAGE

Mouse polyclonal antisera raised against the WCE of *Cb*, *Mk* and rabbit polyclonal antibodies specific for *Cf* *Cf*TX-1 and 2, as well as CSL *Cf* specific AV were tested and compared for their binding against *Cb* WCE proteins.

A robust specificity was consistently observed in the binding of *Cb* specific polyclonal antisera with the partially resolved protein doublet of ~ 43/46 kDa in the *Cb WCE*. In addition three minor specific immune-reactive bands of higher and lower molecular weigth were revealed (**see Figure 3, lane D**). Similarly, *Mk* antibodies cross-reacted strongly in the protein doublet region of ~ 43/45 of the WCE of *Cb* but no other immune-reactive band proteins were detected (**Figure 3, lane F**). Antibodies specific

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to the cytolytic toxins *Cf*TX-1 and -2 cross-reacted weakly with the WCE of *Cb* in the region of 43/46 kDa proteins and reacted strongly with ~ 20 kDa protein (**Figure 3, lane F**).

In a separate study, WCE of both *Cb* and *Cf* were exposed to the CSL *Cf* AV (**Figure 3 Lane H/G** to **I/J** respectively). CLS AV bound to protein bands of an apparent molecular mass of ≥ 32, 43-46, 55 and 65 kDa of *Cb* WCE, with the strongest binding observed to proteins with apparent molecular masses of ~ 43-46 kDa where a doublet of similar size was seen in the *Cf* WCE (**Figure 3, lane I**). The reactive proteins were genuinely bound to the ovine deived CSL AV. Control sheep serum did not react with any of the jellyfish extracts used (data not show)

2.4. Antigenicity of whole nematocyst extracts (native) in immuno-dot-blot assays.

Mouse derived *C. barnesi* polyclonal antibodies reacted most strongly with extracts from homologous species (***Figure 4A***). In contrast, the *Mk* antibodies bound with low affinity to its respective venom though intriguingly, cross reacted with both *Cb* and *Cf* venom (**Figure 4 D-F)**. The four human sera showed different levels of cross reactivity against jellyfish extracts. The human sera that demonstrated greatest cross reactivity was used in the dot blot detection assay against the WCE of the three jellyfish species and the order of cross-reactivity was *Cf* > *Cb* > *Mk*. (see **figure 4 I, G and H**). A different order of cross reactivity was observed when CSL AV was tested against the three jellyfish WCEs. The mice pre-bleeding sera, control sheep sera and serum from a non-stung (naïve) human did not cross react with any WCEs proteins in experiments in which the primary probes reacted against the three jellyfish WCEs. This demonstrated that genuine *specific* binding of experimental antibodies had taken place in the dot blot experiments.

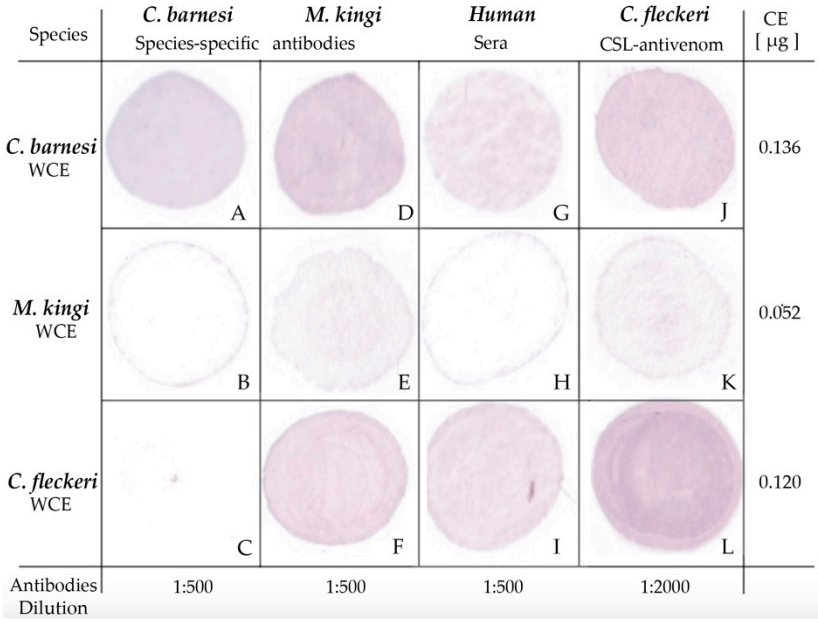

Figure 4 Differential antibody cross-reactivity among jellyfish crude extracts.

Dot blot analysis of jellyfish nematocyst extracts of three cubozoans, *Cb*, *Mk* and *Cf* tested for immune reactivity using four probes: *Cb* and *Mk* polyclonal antibodies raised in mice, human sera and CSL AV. The optimised dilution and the protein concentrations used are indicated. WCE, refers to whole nematocytes crude extracts of nematocyst.

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3. Discussion

This study provides the first insight into *Malo kingi* venom and the comparative utility of a variety of immunological tools potentially available to compare Irukandji and *Chironex fleckeri* venom antigens. Due to limiting quantities of these rare Irukandji venoms, this should be considered a pilot study providing directions for future investigation.

3.1. Preparations of nematocyst extracts

The isolation of *Cb*, *Mk* and *Cf* nematocysts from jellyfish tissue was achieved using a previously described method [66] that has been promoted by several Australian groups [37,64,67,68]. To examine the nematocyst protein content of bell versus tentacular nematocysts, Irukandji box jellyfish samples had to be dissected and grouped in batches of bells and tentacles, followed by the autolysis method for nematocyst isolation that was adapted for small and scarce Irukandji specimens. The nematocysts obtained were cleaned of cellular debris by ultra centrifugation in a discontinuous Percoll gradient [63]. Nematocyst extracts were disrupted with a bead mill homogenizer as used previously for cubozoans [64,67,69]. A constant issue encountered with *Cb* and *Mk* tissue was the small amount of nematocyst material isolated and the adhesion of proteins released following disruption of nematocysts. Special care was required to maximize recovery of nematocyst proteins by repeatly washing the glass beads with extraction buffer and concentration of proteins by TCA precipitation and cold acetone washing of proteins.

3.2. Venom protein profiles

There was no evidence of protein degradation in the venom profiles shown in **Figure 3**. There were significantly fewer protein bands detected in WCE profiles of *Cb* (obtained between this and earlier studies [70,71]. It is important to mention than comparisons with reference 70 (Wiltshire et al XXX, would be very careful perforemed as in that study the specimens identified as *Cb* were not, in fact, *Cb* they are clearly Malo in the photos. Differences in protein components observed in **figures 3C and 3H** for *Cb* are perhaps the result of different lot preparations and mixed stages in the WCE. Discrepancies with other work may be due to methodological differences such as nematocyst extraction protocols, extraction from reconstituted freeze-dried nematocysts instead of fresh preparations, absence of protease inhibitors and subsequent protein denaturation, and resolution of proteins by SDS-PAGE. On the other hand, the nematocyst protein profiles of *Cf* nematocyst proteins, where venom material was not limiting, for example in **Figure 3 lane I** was consistent with previously published reports [64].

3.3. Antigenicity of nematocyst extracts fractionated by SDS-PAGE

Based on the western blot data (**Figure 3, lane D and E**) it is plausible that the apparently immunologically-dominant region ~ 43-46 kDa, observed in *Cb* venom, shares similar antigenic determinants with *Mk* venom. Furthermore, the western blot studies show that the 43-46 kDa, and a smaller ~ 20 kDa protein band of the *Cb* WCE, cross-reacted with the antibodies raised against *Cf* cytolytic toxins CfTX-1 and -2, indicating some probable homology to the Cnidaria pore-forming protein family [35,72]. The stronger binding signal of the ~ 20 kDa protein against the CfTX-1 and -2-raised antibodies may be explained by the possible presence of similar antigens in the two

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different proteins. Whilst this pattern of *Cf* antigen binding has been previously reported by others [65], this is the first assessment of the binding patterns of these reagents against Irukandji venoms.

Several isoforms of CfTX-1 and -2 were reported in a *Cf* nematocyst proteome study [73]. Most recently, nine new pore-forming members were added to the family using a proteo-transcriptomic approach in *Cf* [35]. Hence, in conjunction with confirming the known abundance of pore-forming toxins in *Cf*, this study sought to explore the binding of the polyclonal antibodies raised in rabbit against CfTX-1 and 2, against Irukandji venoms. Based on the binding data presented here, it is plausible that Irukandji jellyfish (*Cb* and *Mk*) may also contain an abundance of pore-forming toxins. This is supported, at least for *Mk*, because in a previous comparative phylogenetic analysis for this Irukandji species, three highly expressed *Mk* transcripts encoding secreted proteins MkTX-A1 (48.55 kDa) ACX30670.1, isoform MkTX-A2 ACX30671.1 and MkTX-B ACX30672.1, were found to be related to the Cnidaria pore-forming toxin family [45]. Moreover, the location of immune reactive bands in the *Cb* WCE, using *Cb* and *Mk* mouse polyclonal antibodies, correlates with the calculated molecular mass of MkTX-A (data not shown).

The strong antibody binding of pore-forming toxins in Cubozoa could be the product of several factors. Firstly, their high abundance in the venom (*i.e.* the dominant region in WCE of *Cb* and *Cf* in the range of apparent molecular weight of 42-46 kDa seen in **figure 3 Lane C and J**), means that, in turn, these proteins dominate the antigen mix innoculated as an immunogen and, secondly, such proteins possess more robust, conserved and exposed antigens, that elicit stronger and/or higher affinity antibody responses in immunized animals. This is in part explained by a recent analysis [48], which indicates that the Cnidaria pore-forming toxin family have evolved under extreme influence of negative selection. This implies that among these toxins there may exist regions of conserved exposed surface regions, subsequently facilitating development of antibodies in exposed animals and potentially resulting in few reactive proteins but which might exhibit strong cross-reactions as observed here.

Conversely, it is likely that potentially medically important, but quantitatively minor constituents of cubozoan venom, that target excitable membranes, may lack antigenic epitopes or display buried epitopes in the native state, impacting on their ability to elicit antibody production in jellyfish venom-sensitized animals, including humans. This should not be misinterpreted, because cubozoan toxins may elicit strong innate immunological responses in envenomated humans independent of T and/or T cell epitopes but may otherwise be molecules acting as 'danger' signals or mimicking molecules involved in immune cell communication [3]. One example is the rapid and uncontrolled production of cytokines that activate T cells and macrophages in what is known as "a cytokine storm" [74]. Cytokines are signaling molecules secreted by a variety of cell types *i.e* immune, epithelial, endothelial and smooth muscle cells and their release may occur in response to pathogens, non-self molecules, and toxins [75]. Interestingly, the symptoms of the cytokine storm include the classic signs of inflammation such as heat, swelling, redness, pain and loss of function. A cytokine storm can also lead to systemic dysfunction of organs [74], similar pathology to that observed in cnidarian envenomation.

3.4. Antigenicity of whole nematocyst extracts (in native state) in dot blots

Although the major elicitors of the cubozoan venom-specific antibody responses are unknown, it is evident that human sera are reactive against jellyfish extracts. The differential human responses towards the venoms of *Cb*, *Mk* and *Cf* could possibly be explained by a distinct pattern of boosting secondary to repeated jellyfish stings in sensitized individuals. For instance, elevated serum levels of IgE are reported to persist for at least four years following jellyfish stings and they may cross-react

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with the venoms of several jellyfish species [76]. Future studies could further map these exposure-antibody relationships.

CSL AV was reactive not only to *Cf* extracts but also to *Cb* proteins and, to a lesser extent, to *Mk* nematocyst proteins. This could be attributed to the different concentration of blotted protein consequent on rarity of *Mk* Irukandji venom. Certainly the results obtained in the dot blot study are consistent with previous results in which CSL AV reacted with jellyfish extracts of cubozoas and also with total proteins extracted from other medusae [70], indicating that despite the venoms having different components, toxicity and potency, there are some shared epitopes eliciting antibody binding. Nevertheless, this cross reactivity as mentioned previously may have a structural, rather than a clinical, significance due to the fact that CSL AV is unable to effectively neutralize the effects of *Cb* envenomation in humans [59].

CSL AV is a complex antibody resource, likely to be produced with diverse venom material (including potentially tentacle tissue and mucus contamination) and degraded proteins. The use of CSL AV remains controversial with reported deaths occurring despite its administration [77]. It has been suggested that it lacks medically important venom components [57]. The percentage of specific nematocyst protein antibodies present in CSL AV may not represent the complete set of venom proteins as a result of low venom protein antigenicity therefore eliciting weak antibody responses in immunized sheep leading to low concentrations of those antigen-specific antibodies. This possibility is supported by the western blot analyses of denatured venom proteins in which only few venom proteins bound CSL AV. In addition, components other than nematocyst proteins (*e.g.* structural components and constituents with a mucus origin) may act as immune-dominant antigens. This is of importance because jellyfish are free living oceanic organisms exposed to a myriad of microorganisms and mucus plays an important role in innate immune defence as the first physicochemical barrier to jellyfish tissue invasion [78].

4. Conclusions

Cubozoans are highly specialized organisms possessing a sophisticated hunting behaviour assisted by an ancestral visual system [79], and venom components shaped by stage specific food preferences [71]. Irukandji and *Chironex fleckeri* venoms are of particular medical significance but have rarely been compared using antibody-based reagents.

In this study species-specific polyclonal antibodies from *Cb* and *Mk* were found to bind an immuno-dominant protein of ~ 43-46 kDa in *Cb* WCEs and this was consistent with the findings when using CSL *Cf* specific AV and also *Cf*TX-1 and 2 specific antibodies. An additional, minor band of 20 kDa was also detected. Collectively these results suggest that *Cb* venom possesses at least three homologous proteins to *Cf*TX-1 and 2, as well as three homologous proteins in *Mk*. Mouse antibodies raised against *Mk* venom cross-reacted with proteins from *Cb*, most likely the paratopes common to epitopes in *Mk*TXA-1, 2 and *Mk*TX-B. Members of this toxin family of porins can cause haemolysis, pain, inflammation, dermonecrosis, cardiovascular collapse and death in experimental animals, suggesting that these toxins are responsible for some of the features of *Cf* envenomation; the homologs in Irukandji may play similar roles [3].

With the exception of a few proteins, not many venom components in Irukandji venoms (*Cb*, *Mk*) were antigenic to the antibody probes used. This suggests that most of these venom proteins are poor antigens. However it is important to bear in mind that the quantity of injected antigen contributes the nature of the response that is evoked; too much or too little antigen may induce suppression, sensitization, tolerance, or other unwanted immunomodulation [80] therefore this may be another reason for the low immune reactivity of venom against polyclonal antibodies. Nevertheless we used 12.5 and 9.1 μg of *Cb* and *Mk* WCE, respectively, that falls within the

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recommended protein concentration range for mice immunization. Further, to enhance the immune response, an adjuvant was used that also assists to preserve the conformational integrity of venom proteins and present the antigens to the appropriate effector cells [81]. Mice control sera did not react at any of the cubozoan venom tested.

This study was constrained by the difficulty in collecting sufficient Irukandji jellyfish to allow a more comprehensive comparative study of jellyfish venoms. Consequently use was made of the more readily available box jellyfish *Cf* venom for developing many of the assays. Despite the lack of definitive experimental resources, it was possible to obtain useful information on the venom proteins of two Irukandji species. Bell and tentacle nematocysts of Irukandji *Cb* differ from each other in protein venom components as demonstrated by their signature pattern on SDS-PAGE. Clearly, despite insufficient *Mk* venom to demonstrate homologous binding of *Mk* specific antibodies, distinct differences emerged between the *Mk* and the *Cb* specific antibodies, suggesting the need for careful dissection of the similarities and differences between Irukandji venoms. The implications of such antibody binding for future antivenom development require further investigation – *Mk* specific reagents may bind both Irukandji and *Cf* venoms.

Future studies should be directed by further refining polyclonal probes as tools in the investigation of cubozoan venoms. It is relevant to highlight the potential use of immune-dominant pore-forming proteins to gain an insight into the immunological mechanisms of box jellyfish toxins, opening the possibility to explore this specific protein family for broad cubozoan immunotherapy. It is possible that these immunogens may produce highly specific antisera to counteract this medically-significant toxin family and for species identification. The results reported here contribute information for rare, yet medically-important jellyfish species.

Materials and Methods

5.1. Collection of samples

Samples of *Cb* were collected at Palm Cove, North Queensland, Australia mostly during daylight hours between 25 December-1st January 2003-2005 using a custom-made net (by Kim Moss, Uninet Enclosure Systems, Cairns, Australia). This was dragged in the water parallel to the beach at a depth of about 1 meter. Juvenile and/or adult medusa samples of *Cb* were either dissected into bells and tentacles or used as a whole to prepare isolated nematocyst (see *below*).

One specimen of an adult *Mk* was obtained during February 2005 offshore of Port Douglas at the northern section of the outer Great Barrier Reef, North Queensland, Australia. Samples of mostly juvenile *Cf* were obtained at different times from November to May 2004-2006 by staff members of Surf Life Saving Queensland during their daily monitoring inside the jellyfish enclosure nets off coastal beaches in Cairns and Townsville, Queensland, Australia.

For protein studies, living *Cb* and *Mk* samples were temporarily stored in 20 litre buckets containing seawater at ambient or air conditioned temperature (~22°C). *Cf* tentacles were exised *in situ* and kept in 1 litre containers with seawater. Samples were processed at James Cook University, Townsville, North Queensland, Australia.

Crude extracts were prepared as described further below in different batch preparations. Cubozoan samples were collected under the Great Barrier Reef Reserve Park permit number: GO7/24733.1.

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5.2. Human sera

Sera were sourced from four healthy adults, all with histories of both Irukandji and *Cf* jellyfish stings and one healthy adult donor without any cnidarian sting history. Whole blood (20 ml) was collected from donors and allowed to clot at 37°C for 1 hr. Samples were transported to the laboratory and incubated overnight to allow the clot to contract. The serum was removed from the clot and any remaining insoluble material was removed by centrifugation at 10,000 g for for 10 minutes at 4°C. The resultant supernatant (designated "serum") was filtered (22 μm) and dispensed in 1 ml aliquots either for immediate use or stored at -20°C until subsequent use.

The blood was obtained from volunteers at the Townsville Hospital and Health Service, under compliance with the National Health and Medical Research Council (NHMRC) "National Statement on Ethical Conduct in Research Involving Humans" (1999) and James Cook University (JCU) Ethics approval number: H2022.

5.3. Polyclonal antibody probes

Polyclonal antibodies were raised in mice against pure whole crude extracts WCE (bell and tentacle nematocyst proteins) of *Cb* and *Mk* in the laboratory of Associate Prof. Heinrich Körner under JCU Ethics Approval Number A936. In brief, nematocyst crude extracts (12.5 and 9.1 μg in PBS, respectively, were mixed and emulsified with complete Freund's adjuvant, and injected subcutaneously into one mouse per jellyfish sample followed by two booster injections, by the same route, of the same protein doses mixed with incomplete Freund's adjuvant at intervals of two weeks. Rabbit polyclonal antibodies specific for *Cf* 43/45 KDa toxins (CfTX-1 and 2) were provided by Dr. Diane Brinkman (Australian Institute of Marine Sciences) but were prepared at the Institute of Medical and Veterinary Science in Adelaide, as previously described [65]. *Cf* specific ovine antibodies were purchased from the commercial box jellyfish antivenom manufacturer (CSL Ltd, Australia).

Whole rabbit sera, mice sera and antivenom were used in this study. No fractionation of immunoglobulins was performed.

5.4. Preparation of venom extracts

Nematocysts were obtained from jellyfish using the autolysis method described previously [66]. Cellular debris was subsequently removed by ultracentrifugation in Percoll according to standard protocols [63]. Packed undischarged nematocysts were placed in a nematocyst extraction buffer consisting of water containing protease inhibitors at a final concentration of 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10μM phenylmethane sulfonyl fluoride (PMSF), 1 μM leupeptin and 1 μM pepstatin. Nematocyst samples were disrupted with glass beads in a mini bead beater, under cold conditions until complete nematocyst discharge was confirmed by light microscopy. Bell and tentacle nematocyst extracts were obtained by dissecting *Cb* tentacles and bells from 5 mostly juvenile specimens and performing separate extractions. WCE of *Cb* were prepared with either 5 adults or a mixed pooled batch of mostly juveniles. Only one whole adult specimen of a mature *M. kingi* WCE was available to obtain a WCE.

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586 *5.5. Protein electrophoresis*

587 Proteins from crude extracts were precipitated in 10% trichloroacetic acid (TCA) and cold
588 acetone followed by centrifugation at 15,000 rpm for 15 min and a final wash with cold acetone.
589 Protein concentrations were determined at 280 nm using bovine serum albumin (BSA) as a standard
590 and solubilized in sodium dodecylsulphate (SDS) sample buffer [82], containing β-mercaptoethanol
591 and denatured at 95ºC for 5 minutes. 5-10 µl (8.3 x 7.3 cm gels) or 10-15 µl (large gels 16 x 20 cm) of
592 different protein amounts were resolved by SDS (12.5-15%) polyacrylamide gel electrophoresis
593 (SDS-PAGE) as described previously [83] using a mini-Protean II, Bio-Rad for *Cb* and a PROTEAN
594 IIxi electrophoresis system, Bio-Rad was used for *Cf* samples.

595

596 Apparent molecular masses were determined by comparing protein mobilities with protein
597 standards [84] (5 µl of either pre-stainded molecular mass marker (NEB), Page-Ruler protein SM0671
598 or BenchMark™ Protein Ladder from Invitrogen). Proteins bands were visualized by Coomassie
599 blue R-250 staining [82]. Images of denatured venom profiles on polyacrylamide were analysed with
600 the open software ImageJ [85]

601

602 *5.6. Western blot (WB) and dot-blot (DB) analysis*

603 Nematocyst proteins resolved by SDS-PAGE were electrotransferred (mini-Trans-Blot, Bio-Rad)
604 to nitrocellulose membranes. For DB assays, venom extracts were spotted onto nitrocellulose
605 membranes, free binding sites were blocked with 5% (w/v) skim milk in a solution of Phosphate
606 Buffered Saline with 0.1 % of Tween® 20 (PBST) for 1 hr at room temperature. Western blot analysis
607 was performed using previously titrated and diluted CSL ovine antivenom (1:2500), human sera
608 (1:500), *Mk* or *Cb* polyclonal antibodies raised in mice (1:100-500).
609
610 Negative controls: sera from a healthy adult subject without Cnidaria envenoming episodes,
611 normal sheep serum, as well as mouse and rabbit pre-bleedings were used. Primary antibodies were
612 detected using anti-rabbit, anti-sheep, anti-human or anti-mouse alkaline phosphatase [28]
613 [28]-conjugated antibodies (diluted 1:4000-5000).
614
615 Antigen-antibody binding was detected with nitro blue tetrazolium and 5-bromo-4-chloro-
616 3-indolyl phosphate -NBT/BCIP (Promega).
617
618

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Supplementary Materials: Figure S1

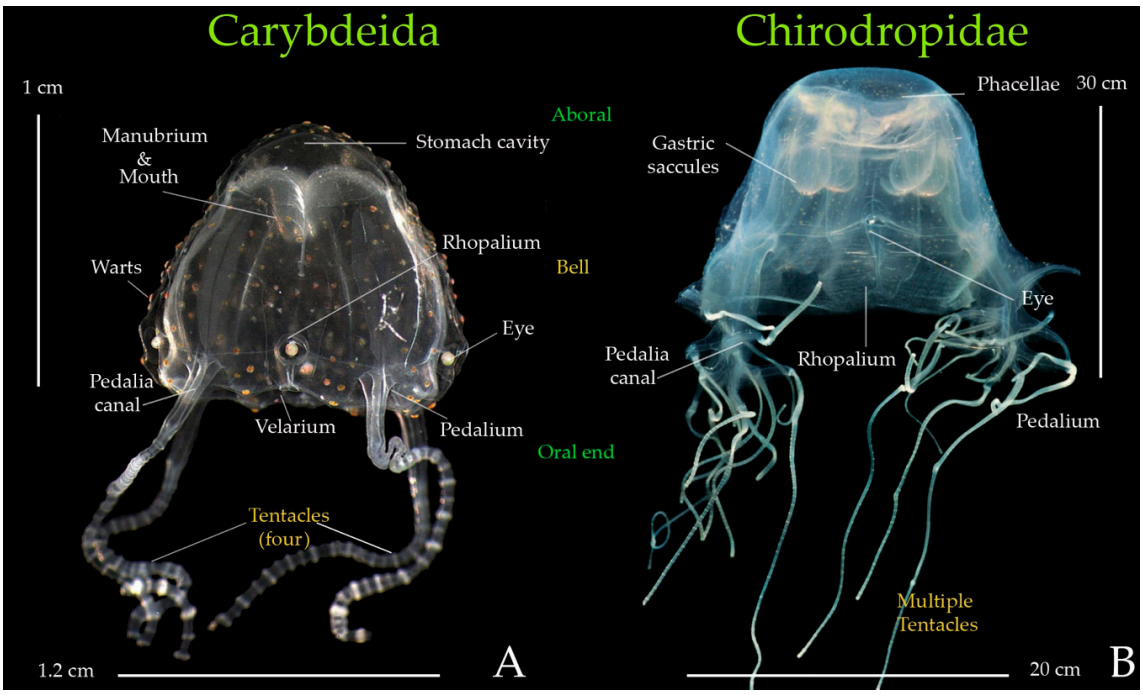

Figure S1 Morphological differences between the Cubozoa orders Carybdeida and Chirodropida. In **A,** Mature specimen of *C. barnesi*; in **B,** *Chiropsalmus quadrumanus* (Photo B, copyright of Alvaro Migotto and used with permission)

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Conceptualization, G.A.S and J.N.B.; Methodology, N.B., K.W., L.A.G. and G.A.S.; Software, G.A.S and J.N.B.; Validation G.A.S., K.W., L.A.G. and J.N.B.; Formal Analysis, G.A.S.; Investigation, G.A.S., K.W., L.A.G. and J.N.B.; Resources, G.A.S., K.W., L.A.G. and J.N.B.; Data Curation, G.A.S,; Writing-Original Draft Preparation and Writing-Review & Editing, J.N.B., K.W., L.A.G. and G.A.S. Visualization, Supervision, Project Administration, Funding Acquisition G.A.S., K.W., L.A.G. and J.N.B. Field trips were executed by L.A.G and G.A.S.

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