

A COMPARISON OF THE MUSCULAR ORGANIZATION OF THE RHOPALIAL STALK  
IN CUBOMEDUSAE (CNIDARIA)

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## ABSTRACT

In the phylum Cnidaria, little is known about the organization of sensory systems of the motile forms, jellyfish. Cubomedusae, or box jellyfish, have four sophisticated sensory structures (rhopalia), one on each side of the box-shaped bell. Each rhopalium contains a statolith, two complex eyes, four ocelli and sensory epithelia, and is suspended by a stalk within a cavity (niche) that opens on the outside of the bell. The function of the stalk, beyond forming a connection between the rhopalium and the bell, is largely unknown. Electron microscopy and light microscopy were utilized to determine the function of the rhopalial stalk and compare stalk morphology among four species of box jellyfish (Cnidaria: Cubozoa), with an emphasis on muscle fiber organization. A branch of the gastrovascular cavity forms the center of the stalk surrounded by the gastrodermis, mesoglea and epithelium. Epitheliomuscular cells make up the majority of the epithelium. Muscle fibers are smooth, longitudinal and asymmetrically arranged around the periphery of the stalk. In three out of four species, the stalk bends or shortens in response to potentially injurious stimuli to move the rhopalium into a more protective position. The structure of the rhopalial niche varies in each species and can be correlated to muscle fiber percent area.

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## DEDICATION

This thesis is dedicated to my three nieces, Savannah Dalayne, Madalyn Opal and Kaitlyn Rose Elizabeth. Always follow your dreams and remember that the future is in your hands. May God bless you the strength, courage and wisdom to do His will.

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## INTRODUCTION

The phylum Cnidaria is made up of four separate classes, Anthozoa (corals and anemones), Scyphozoa (the larger, more conspicuous jellyfish), Hydrozoa (small jellyfish, *Hydra*, and hydroids) and Cubozoa (box jellyfish). The corals and anemones are the only members of the phylum that are found in the polyp form only, while the other three classes have a life cycle that includes polyp and medusa forms (Parkefelt et al. 2005).

### **Cnidarian morphology**

Cnidarians exhibit biradial symmetry, oriented along the aboral-oral axis. The subumbrella forms the disc, or the oral surface, and the exumbrella, forms the aboral surface. The mouth is the only opening to the digestive system and is found at the tip of a manubrium (Ruppert et al., 2004). The body wall consists of three layers: the epidermis, the mesoglea and the endoderm (gastrodermis). Only the epidermis and gastrodermis are composed of living tissue while the mesoglea is a gelatinous layer, situated between the epidermis and gastrodermis. The mesoglea is mostly acellular, although it may contain amoebocytes that may play a role in digestion, wound repair and antibacterial defense (Pechinik, 2000), and is essentially a connective tissue. Cnidocytes, muscle, nerve, interstitial, glandular, and ciliated cells are found in the epidermis and gastrodermis although they may have different functions. Germ cells are normally found in the gastrodermis, while sensory cells are restricted to the epidermis (Ruppert et al., 2004).

Cnidarians are also unique in that they are one of the first eumetazoans to have evolved a nervous system (Garm et al., 2006). Communication in the nervous system is by chemical synapses (including neuromuscular junctions) and gap junctions (Hydrozoa only). Polyps contain only nerve nets, which are diffuse, non-directional forms of signal conduction. Two

nerve nets are found in polyps and medusae, one nerve net each in the base of the gastrodermis and epidermis, and are joined via interconnections that bridge the mesoglea. In addition to these nerve nets, medusae have nerve rings and ganglion-like structures. These “ganglia” are located on the periphery of the bell and are associated with sensory organs such as mechanoreceptors, chemoreceptors, and photoreceptors (Ruppert et al., 2004).

Sensory structures of Cubomedusae and Scyphomedusae are concentrated in the rhopalia, which contain the statoliths and ocelli, as well as the pacemakers that control swimming. The statolith has been associated with balance and body position relative to gravity. The simple light receptors, the cup-shaped ocelli, are not capable of forming an image, and contain a light sensitive pigment (Pechnik, 2000).

Little is known about the structure and function of the sensory structures of box jellyfish, although sophisticated behaviors are associated with such organs. For example, Cubomedusae vertically migrate, form breeding aggregations (Hamner, 1995) and navigate away from obstacles.

### **Class Cubomedusa**

Cubomedusae are the class with the fewest species in Cnidaria (Parkefelt et al. 2005), and were once considered to be part of the Scyphomedusae. However, recent evidence suggests that they deserve class status (Werner 1973; Werner et al. 1976; Coates 2003). Cubomedusae differ from other cnidarians in their body structure, choice of habitat, observed behavior patterns, and nervous and sensory systems (Lawton 2003; Coates 2004; Parkefelt et al. 2005). They also differ from scyphozoans as the polyp stage in the life cycle does not strobilate, instead producing one medusa per polyp (Pechnik, 2000).

There are two families of box jellyfish, the Chirodropidae and the Carybdeidae, that differ in the number of tentacles projecting from each pedalium, a muscular fleshy pad located at the corner of the bell. Species of the family Chirodropidae have four pedalia, one on each corner, each with multiple tentacles. In the Carybdeidae, each tentacle is connected to a single pedalium, with one exception, *Tripedalia cystophora*, which usually has two or three tentacles connected to one branched pedalium (Kramp, 1961; Collins, 2002; Coates, 2004).

Cubomedusae are efficient, agile swimmers (Stewart, 1996; Satterlie et al., 2005) that have the ability to change direction in just a few swim contractions (Satterlie et al., 2005) in response to photic or visual stimuli (Satterlie, 2002), and can actively avoid obstacles in their environment (Ikeda et al., 2000; Parkefelt et al., 2005). They are normally found in near-shore habitats in tropical and subtropical waters, such as the coastal water off sandy beaches, mangroves, kelp forests and coral reefs (Coates 2003; Parkefelt et al. 2005). Cubomedusae actively select their preferred habitat as a result of their swimming abilities, and are avid predators (Larson, 1976; Buskey, 2003; Parkefelt et al., 2005). They have been observed to “sleep” at night (Lawton, 2003; Parkefelt et al., 2005) and some species even engage in copulatory behavior during mating, resulting in internal fertilization (Stewart, 1996; Studebaker, 1972; Parkefelt et al., 2005). Box jellyfish elicit a protective behavior, termed crumpling, in response to potentially injurious stimuli. This behavior involves the contraction of all four tentacles (or sets of tentacles) and the inward curling of the tentacle pedalia and subumbrellar margin. This behavior can be observed asymmetrically during feeding where only a single tentacle is brought into the bell for a transfer of prey from the tentacle to the manubrium (Satterlie et al., 2005).

## **Cubomedusae anatomy**

The bell of the box jellyfish is about as tall as it is wide and exhibits radial symmetry around the oral-aboral axis. The velarium is an inward folding of the swimming bell at the margin, (Ruppert et al., 2004) and serves to increase cubomedusan swimming efficiency by restricting the size of the bell aperture. Four rhopalia are found in cubomedusae, one in the center on each side of the box-shaped bell (Satterlie, 2002).

The rhopalia are sophisticated sensory structures that lie in small cavities (rhopalial niche) that open to the outside of the bell near the bell margin. They are suspended by a rhopalial stalk within this cavity (Parkefelt et al., 2005). A segment of exumbrellar tissue, or hood, incompletely covers the opening of the niche and may serve a protective function in shielding the rhopalia as it lies in the indented exumbrellar niche. The rhopalial niche opening varies from species to species and may be useful in identifying species (Hartwick, 1991).

Each rhopalium contains a statolith, two complex eyes, up to four pigment ocelli, and sensory epithelia (Satterlie, 2002). The statolith is a crystalline concretion that has been proposed to act as a gravity-sensing device, as well as to insure that the eyes are constantly oriented in the proper direction relative to gravity (Berger, 1900; Coates, 2004; Parkefelt et al., 2005). One small upper lens eye and one large lower lens eye (complex eyes) are placed along the median line of the rhopalium, with the slit ocelli found just above and on either side of the large lens eye. The pit ocelli are found on either side of and behind the small lens eye. The eyes face inward, looking through the bell tissue. This visual organization allows for a horizontal and vertical field of view near 180° for each rhopalium, and the transparency of the bell allows for multidirectional vision. Together, the rhopalia provide a near complete view of the surroundings (Coates, 2004; Parkefelt et al., 2005). Removal of all rhopalia robs cubomedusae of the ability

to produce spontaneous swimming contractions as the pacemakers for swimming movements are found in the upper region of each rhopalium. However, if three rhopalia are removed and the fourth is left intact, swimming will continue until the final rhopalium has been removed (Satterlie, 2002).

### **Rhopalial stalk**

Garm et al. (2006) found the rhopalial stalk of *Tripedalia cystophora* to be circular in cross-section with a monolayered epidermis. In transverse section, the gastrovascular cavity is at the center, surrounded by the gastrodermis, the mesoglea, and the epidermis. Satterlie et al. (2005) found muscle fibers within the rhopalial stalk of *Tripedalia cystophora* that stain in actin-immunohistochemistry (IH) preparations, with a narrow band of actin-IH-positive fibers in the oral wall of the stalk only. This band splays out and terminates just above the attachment of the stalk and subumbrella (Satterlie et al., 2005). Additionally, Hartwick (1991) noted active muscular control of the rhopalia of *Carybdea sivickisi*, and suggested that the musculature in the stalk may permit vision both adaxially and abaxially. As the eyes of the cubozoans are oriented inwards, the muscle in the rhopalial stalk may function in guiding the eyes to obtain an image from a particular region via the opening of the niche (Hartwick, 1991).

A nerve ring runs from each pedalum to the origin of the rhopalial stalk and then loops back down to the next pedalial base, continuing around the subumbrella in this manner. Each rhopalial stalk contains neuronal networks connecting it to the subumbrellar nerve ring (Satterlie, 2002). Extracellular physiological recordings from the rhopalial stalk have revealed a pacemaker signal coming from the rhopalia, which directly controls swimming activity (Satterlie and Spencer, 1979; Satterlie and Nolen, 2001; Satterlie, 2002; Garm et al., 2006). Garm et al. (2006) also found significant neural activity in the rhopalial stalk of *Tripedalia cystophora*

indicating that the conduction of visual signals must occur within the ring nerve and the rhopalia, and is transmitted by the stalk to the nerve ring. This supports the contention that the two major components of the central nervous system in cubomedusae include the ring nerve and rhopalia (Garm et al., 2006). Although the nervous system of Cnidarians is usually considered “simple”, box jellyfish essentially have sophisticated integration centers (rhopalia) that are similar to the ganglia of higher invertebrates.

Presented here is a morphological description of the rhopalial stalk in four species of box jellyfish with an emphasis on the asymmetry of muscle filament distribution to determine if there is a morphological basis for turning or twisting the rhopalium. The presence of helical or circular muscle would indicate a twisting of the rhopalium, while the presence of only longitudinal muscle would imply shortening or bending. Rather than twisting the rhopalium, the function of the muscle may be to move the rhopalium upward into a more protected position in the rhopalial niche or to bend it away from the niche opening. The stalk is an important structure as it is the only connection between the rhopalium and subumbrellar nervous structures. Therefore, any signal that is being shared between the rhopalium and the ring nerve is sent through the stalk.

Longitudinal myofibrils in the stalk epidermis are positive for phalloidin and anti-actin immunohistochemistry. Additionally, the stalk is shown to contain both an epidermal nerve and a mesogleal nerve. Statistical analyses show a difference in muscle fiber distribution within each species and within different regions of the stalk. The rhopalial niche differs between species and may be correlated with muscle fiber distribution.

## METHODS

### **Specimen collection**

Four species were chosen for their availability, phylogeny and habitat. *Chiropsalmus quadrumanus* and *Carybdea alata* were obtained from trawls in approximately 20-30 ft of water off the coast of Wilmington and Southport, North Carolina. Bell diameter ranged from 6 to 8 cm for *Chiropsalmus*, and from 1 to 2 cm for *Carybdea alata*. *Carybdea marsupialis* was hand-collected by divers off the coast of Santa Barbara, California. Bell diameter ranged from 4 to 6 cm. *Tripedalia cystophora* specimens were hand-collected from Puerto Rico. All animals were delivered to the Center for Marine Science, Wilmington, NC where they were kept alive in a holding tank until fixation. *Carybdea* sp. and *Tripedalia cystophora* belong to the family Carybdeidae, while *Chiropsalmus quadrumanus* belongs to the family Chirodropidae. *Carybdea* sp. and *Chiropsalmus quadrumanus* are all found along sandy beaches, while *Tripedalia cystophora* is found among the mangrove roots of Puerto Rico. *Carybdea* sp. and *Chiropsalmus quadrumanus* were first observed to document the structure of the rhopalial niche with the Sony Handycam DCR-DVD201 or a World Precision Instruments digital eyepiece camera.

### **Transmission Electron Microscopy (TEM)**

A few specimens were anesthetized in a 1:2 ratio of MgCl<sub>2</sub> (0.33 M) to seawater, the rhopalia or rhopalium niche dissected, briefly rinsed in seawater and placed in fixative. *Tripedalia cystophora* was fixed using the first protocol described below, while the latter fixations were used for *Carybdea* species and *Chiropsalmus quadrumanus*.

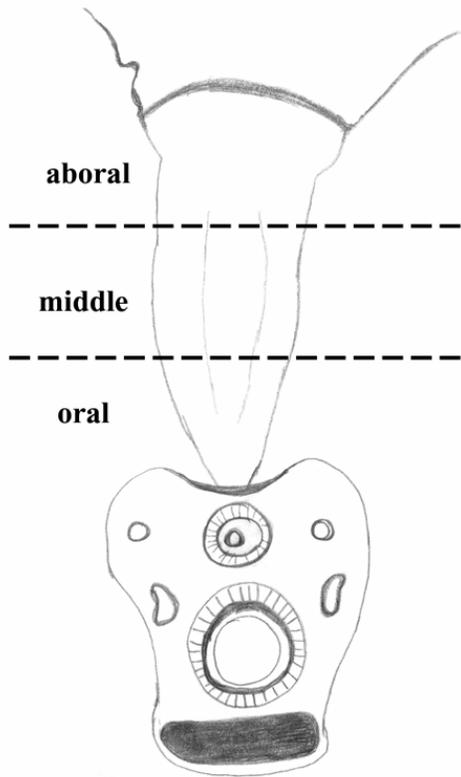
*T. cystophora* specimens were fixed using 2.5% glutaraldehyde in 0.4 M Millonig's phosphate buffer (pH 7.6; Millonig, 1961), postfixed in 4% osmium tetroxide in 1.25% sodium

bicarbonate (1 hr), dehydrated in a graded series of ethanol and propylene oxide, transferred to Spurr's epoxy resin (Spurr, 1969) and embedded in resin. *Carybdea* sp. and *Chiropsalmus quadrumanus* individuals were fixed over two to five days at 4°C in a combination of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M Millonig's phosphate buffer (pH 7.6), transferred to 0.15 M Millonig's phosphate buffer (Millonig, 1961), postfixed in 1% osmium tetroxide in 1.25% sodium bicarbonate (1 hr), dehydrated in a graded series of ethanol, and embedded in Spurr's resin (Spurr, 1969).

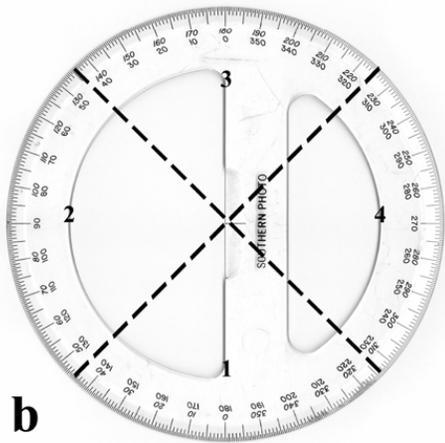
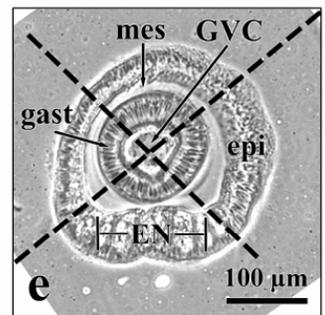
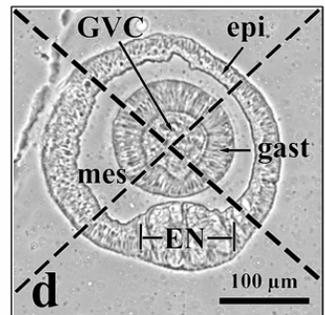
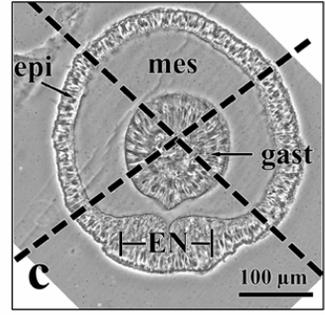
Thick sections were cut on the Sorvall MT2-B Ultra microtome for determination of orientation of tissue and for area measurements. Thin sections, 90 to 100 nm, were cut with a diamond knife on the Reichert-Jung Ultracut E ultramicrotome, picked up with Formvar coated grids and stained with 2% uranyl acetate and lead citrate (Reynolds, 1963). Grids were examined with a Philips CM 12 TEM and micrographs taken with a 3 1/4" x 4" plate camera on Kodak EM 4489 Film. The film was processed and the negatives scanned with a Microtek scanner. Micrographs were digitally processed with Adobe Photoshop 7.0.

Longitudinal sections of the stalk were used to determine orientation and type of muscle fibers in the stalk. For analyses of muscle density, approximately five animals of each species were used, with the exception of *T. cystophora*, where only two animals were used due to limited availability. Cross-sections were taken at the aboral, middle and oral regions of the stalk (Figure 1), except for *T. cystophora* where only the middle and oral regions were sampled. The aboral region was eliminated as the tissue had been fixed in epoxy resin and sectioned prior to sampling. Quadrants were identified on plastic sections where the first quadrant was centered on the thickest part of the epidermis (containing the epidermal nerve), and the thinnest part of the mesoglea. Quadrants were assigned clockwise from this starting point in a cross-hair

**Figure 1.** The orientation of the rhopalial stalk of the box jellyfish. (a) The stalk was divided into three regions: aboral (c), middle (d) and oral (e). A protractor was used to ensure constant orientation during sampling and vector analysis (b). Cross-sections of the stalk (c,d,e) were divided into four quadrants (b), where the thickest part of the epidermis (epi) was in the center of quadrant 1 at 0°. EN - epidermal nerve; mes – mesoglea; gast – gastrodermis; GVC – gastrovascular cavity.



**a**



**b**

pattern, resulting in a total of four quadrants. At least five samples were taken from each quadrant for each region of the stalk. Muscle fibers were identified and selected in Adobe Photoshop 7.0 and the area ( $\mu\text{m}^2$ ) coverage measured in Image ProPlus 6.0. Muscle fiber area was divided by the total area of the stalk (as measured in Image ProPlus 6.0) and the total area of each sample's respective quadrant. Log-transformed data was then analyzed in Statistica 6.0 (StatSoft). Standard ANOVA's and MANOVA's were conducted to find significant differences. The Tukey HSD (Honest Significant Difference) post-hoc test was used when significant differences were found.

Vector analysis was used to determine putative direction of movement of the stalk by assigning a scale for values of muscle percent area ( $0.00015 \mu\text{m} = 1 \text{ cm}$ ). Vectors were then drawn within quadrants on a protractor in Adobe Photoshop 7.0. The zero of the protractor was in the center of quadrant 1, and angles determined clockwise from this point. Therefore, the center of quadrant one was at  $0^\circ$ , the center of quadrant 2 at  $90^\circ$ , etc. Directional vectors were subtracted from each other, forming a rectangle from which the angle could then be determined, thus providing a putative direction of contraction, but only if the muscle fibers contract at once.

### **Confocal microscopy**

Two fixation protocols were used to identify and label muscle fibers. Both phalloidin and anti-actin were utilized to determine the orientation and location of muscle fibers in the stalk. Dissected rhopalial niches were placed in 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4) and fixed for at least four hours to overnight. Tissue was washed with 0.01 M phosphate buffer (pH 7.4) with detergent (0.05% Tween 20) to increase permeability of the tissue, dehydrated/rehydrated with ethanol (ETOH), and incubated in goat serum (2-4 hrs). The tissue was then incubated in rhodamine-labelled phalloidin (Molecular Probes) or rabbit anti-actin

(Sigma #A 5060), for 24-48 hours in the cold. Samples labeled with phalloidin were then washed in phosphate buffered saline (PBS, pH 7.4) and cleared in a glycerine-based mounting medium (9 parts glycerol to one part 50 mM Tris buffer, pH 9). Anti-actin samples were washed in PBS, incubated in for 12-24 hours in secondary antibody (anti-rabbit FITC; 12 hours), washed in PBS (pH 7.4) and cleared in mounting medium.

Alternatively, dissected rhopalia were fixed in 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.2) overnight to three days at 4°C. Samples were washed in 0.2 M Millonig's buffer (pH 7.2) at room temperature, rinsed in 0.4 M glycine (pH 7.2, one hour), washed in 0.25% Triton X-100 in 0.1 M Millonig's buffer (pH 7.2) and incubated (24-48 hours) at 4°C in phalloidin or anti-actin, diluted in 0.1 M Millonig's buffer (pH 7.2; Millonig, 1961) with 0.25% Triton X-100 and 0.25% goat serum. Phalloidin samples were then rinsed in 0.1 M Millonig's buffer (pH 7.2; Millonig, 1961) at room temperature before clearing in mounting medium. Anti-actin samples were washed in 0.1 M Millonig's buffer (pH 7.2) containing 0.25% Triton X-100 before being incubated overnight in anti-rabbit FITC in 0.1 M Millonig's buffer, pH 7.2 (Millonig, 1961), containing 0.25% Triton X-100 and 0.25% goat serum. Samples were washed in 0.1 M Millonig's buffer (pH 7.2; Millonig, 1961) and cleared in mounting medium. All samples were stored in the cold and optical sections and 3-D reconstructions of labeled tissue were obtained with an Olympus FV 1000 confocal microscope.

## **Histology**

Histological sections provided a general morphological description of the stalk and its position in the rhopalial niche. Dissected rhopalial niches were placed in either 4% paraformaldehyde in 0.2 M Millonig's buffer (pH 7.6; Millonig, 1961) over a series of 3-4 days at 4°C, or were fixed in 4% paraformaldehyde in phosphate buffered saline (pH 7.4) for four

hours to overnight. Both fixations yielded similar results with no obvious differences. Tissue was then rinsed in appropriate buffer, a series of washes in distilled water (DI), and dehydrated in a graded series of ethanol – 70%, 85%, 100%, 100% - followed by two changes of 100% xylene. Tissue was placed in ½ xylene: ½ paraffin in the oven (56°C) for one hour, and then straight paraffin overnight (56°C) before embedding in plastic molds. Serial sections were cut at 8-10 µm in either the longitudinal or transverse plane.

Slides were stained following the one-step trichrome method (Gabe, 1968). Paraffin was removed with xylene and the sections hydrated by a descending series of ethanol, deionized water and at least 10 minutes in the trichrome stain. After staining, slides were quickly rinsed with DI and transferred to 100% ethanol (2 changes), followed by two changes of xylene. In the one-step trichrome stain, nuclei, muscle and some secretion granules are red; cytoplasm appears pale pink or gray; collagen fibers and some secretion granules are green; and nerves, blue-gray (Gabe, 1968). Slides were observed with a standard compound microscope. Photographs were taken on an Olympus BX60 light microscope equipped with a SPOT RTKE digital camera (Diagnostics, Inc.).

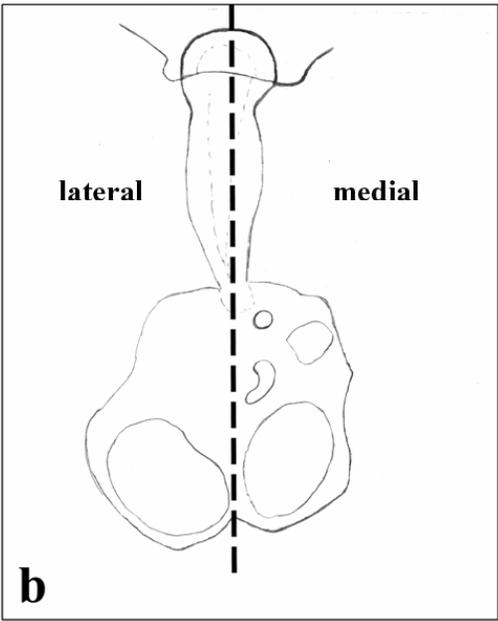
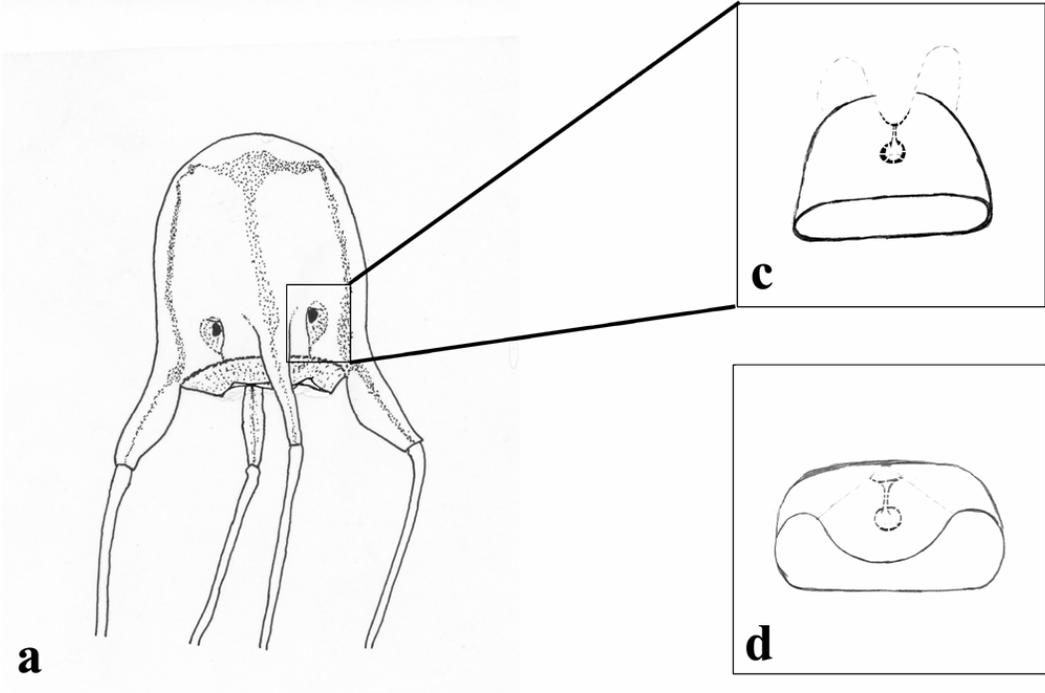
## RESULTS

The rhopalial stalk of the box jellyfish is enclosed within a rhopalial niche, one on each side of the box-shaped jellyfish. The rhopalial niche opening is different from species to species and can be used as an additional source of species identification (Figure 2). Attached to the roof of the niche, the rhopalial stalk connects to the peripheral side of the rhopalium at the oral end. Stalk length differs in each species, ranging from 0.280 cm ( $\pm 0.024$ ) in *Carybdea alata* to 0.430 cm ( $\pm 0.02$ ) in *C. marsupialis* (Table 1). Data for *Tripedalia cystophora* is not given as no live animals were available to measure stalk length, bell height and diameter. In each species, the stalk is nearly circular in cross-section with a branch of the gastrovascular cavity at the center of the stalk, surrounded by the gastrodermis, mesoglea, and epidermis (Figure 1). Stalk area and layer thickness vary from species to species (Table 2).

### **Rhopalial niche structure**

The structure of the rhopalial niche was documented in *Carybdea marsupialis*, *Carybdea alata* and *Chiropsalmus quadrumanus* (Figure 2). No live animals of *T. cystophora* were available to determine the structure of the rhopalial niche. In *Carybdea alata*, the opening is small, slit-shaped measuring approximately 0.47 mm tall by 2.92 mm wide. The heart-shaped opening of *C. marsupialis* measures approximately 2.23 mm wide and 3.96 mm across. A hood hangs down over the opening, serving as a protective barrier. The opening in *Chiropsalmus quadrumanus* is wider than it is tall, approximately 2.81 mm by 0.33 mm. A fold of tissue curves down in the center on the top of the niche to form a shape that is taller on either end than in the middle.

**Figure 2.** The rhopalial niche structure of the box jellyfish. (a) The box jellyfish is about as tall as it is wide, with four rhopalia, one on each side of the bell. The rhopalium is suspended by a stalk within the rhopalium niche. (b) The eyes face inward on the medial side of the rhopalium, through the center of the bell. (c) The rhopalial niche of *Carybdea alata* is small, and slit-shaped measuring approximately 0.47 mm tall by 2.92 mm wide. (d) In *Chiropsalmus quadrumanus*, the rhopalial niche is wider (2.81 mm) than it is tall (0.33 mm), with a hood in the center of the opening. (e) The rhopalial niche opening of *Carybdea marsupialis* is heart-shaped measuring approximately 2.23 mm wide and 3.96 mm tall.



**Table 1.** Bell height (cm  $\pm$  SEM), diameter (cm  $\pm$  SEM) and stalk length (cm  $\pm$  SEM) for *Carybdea marsupialis*, *Chiropsalmus quadrumanus*, and *Carybdea alata*. *Tripedalia cystophora* was not included as no live animals were available to measure stalk length, bell height and bell diameter.

Species	Bell Height (cm) $\pm$ SEM	Bell Diameter (cm) $\pm$ SEM	Stalk length (cm) $\pm$ SEM
<i>C. marsupialis</i> (n=8)	1.50 $\pm$ 0.05	5.38 $\pm$ 0.43	0.430 $\pm$ 0.021
<i>C. quadrumanus</i> (n=20)	1.59 $\pm$ 0.13	7.13 $\pm$ 0.32	0.400 $\pm$ 0.048
<i>C. alata</i> (n=7)	2.64 $\pm$ 0.33	1.18 $\pm$ 0.118	0.280 $\pm$ 0.024

**Table 2.** Cross-sectional stalk area (cm + SEM) and size range (cm) of the epidermis, mesoglea and gastrodermis for *Carybdea alata*, *C. marsupialis*, *Chiropsalmus quadrumanus* and *Tripedalia cystophora* in each region. Only the middle and oral region for *Tripedalia cystophora* is shown as the aboral region was not sampled. The latter three columns provide approximate ranges of size (cm) for each tissue layer in each region for all species. The range of each tissue layer illustrates the size variance of the epidermis and mesoglea, although the gastrodermis is relatively homogenous in size throughout the stalk for each species. The large range of the epidermis and mesoglea is due to the thickening of the epidermis and the thinning of the mesoglea in the vicinity of the epidermal nerve.

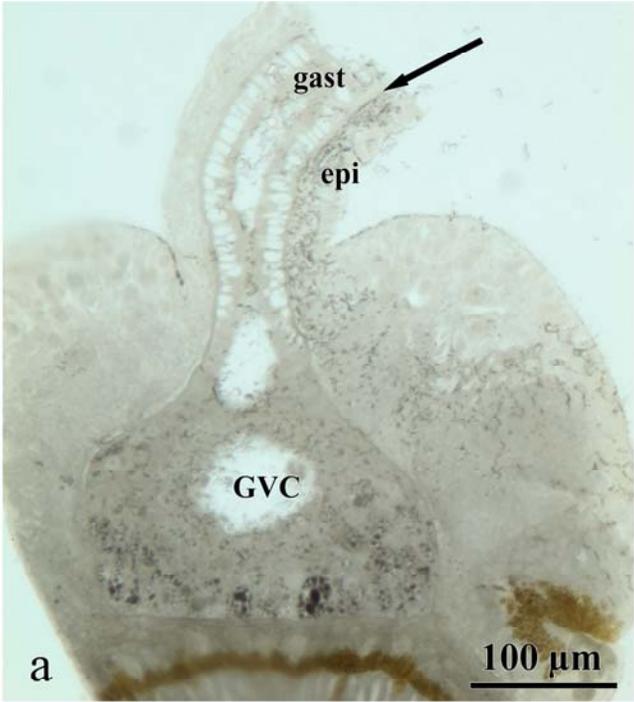
Species	Region	Stalk Area (cm ± SEM)	Epidermis (cm)	Mesoglea (cm)	Gastrodermis (cm)
<i>C. alata</i>	Aboral	23.40 ± 8.14	0.027 – 0.064	0.021 – 0.065	0.044 – 0.055
	Middle	18.10 ± 11.67	0.028 – 0.059	0.010 – 0.024	0.043 – 0.049
	Oral	5.67 ± 0.47	0.037 – 0.064	0.006 – 0.029	0.037 – 0.044
<i>C. marsupialis</i>	Aboral	8.07 ± 0.36	0.08 – 0.026	0.013 – 0.039	0.026 – 0.040
	Middle	5.66 ± 2.34	0.005 – 0.030	0.016 – 0.072	0.020 – 0.031
	Oral	7.64 ± 1.60	0.008 – 0.026	0.016 – 0.037	0.034 – 0.040
<i>C. quadrumanus</i>	Aboral	16.82 ± 8.59	0.016 – 0.056	0.004 – 0.042	0.028 – 0.057
	Middle	9.38 ± 4.11	0.013 – 0.046	0.007 – 0.032	0.039 – 0.056
	Oral	28.84 ± 6.97	0.019 – 0.068	0.002 – 0.030	0.040 – 0.055
<i>T. cystophora</i>	Middle	2.69 ± 0.60	0.007 – 0.015	0.003 – 0.007	0.010 – 0.012
	Oral	0.96 ± 0.27	0.009 – 0.019	0.002 – 0.005	0.010 – 0.012

## **Rhopalial Stalk**

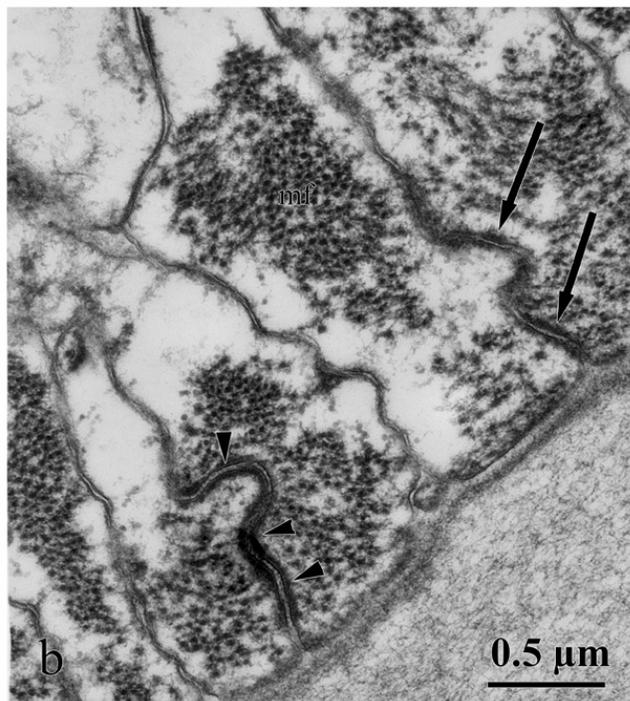
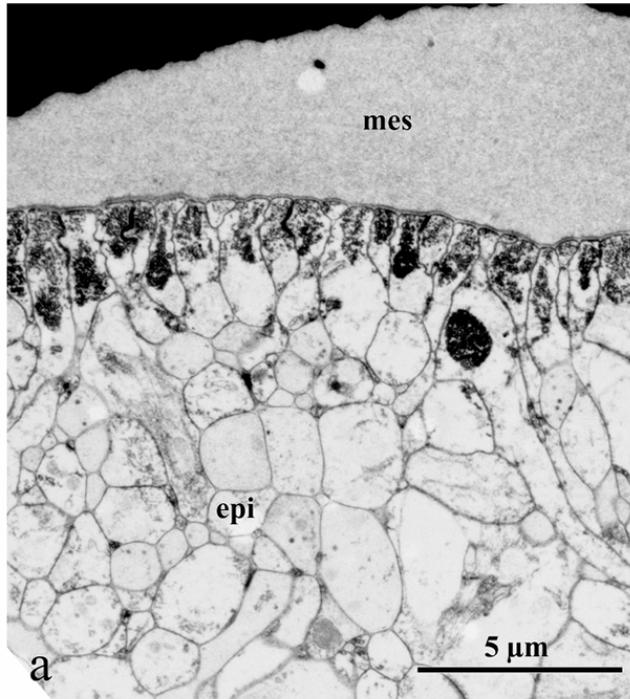
The morphology of the rhopalial stalk was investigated with light, confocal and transmission electron microscopy (TEM). The stalk is circular in cross-section and consists of a single layer of epitheliomuscular cells (Figure 3 a, b) surrounding the mesoglea, the gastrodermis and a branch of the gastrovascular cavity. The epithelial cells stain red in Gabe's trichrome indicating the presence of muscle, nuclei and secretion granules. Epithelial cells are coupled via septate junctions and desmosomes (Figure 4). No evidence of gap junctions was found throughout the tissue of the stalk.

The proximal  $\frac{1}{4}$  of the stalk epithelium is composed of epithelial cells, void of any muscle fibers, while the distal  $\frac{3}{4}$  contains epitheliomuscular cells. The epithelial cells found at the most aboral end of the stalk consist of a nucleus, pigment granules, and some mitochondria. Microvilli are often found at the apical end of the cell. The epitheliomuscular cells in the more distal part of the stalk contain a vacuole, nucleus, pigment granules at the more apical end of the cell with muscle fibers at the basal end of the cell (Figure 5). The smooth muscle fibers run in the aboral-oral axis (longitudinal to the stalk) and are organized into myonemes (Figure 6). Longitudinal sections through the stalk indicate the presence of only this longitudinal muscle, measuring approximately 5  $\mu\text{m}$ , and the absence of either oblique or circular muscle that would create a twisting or turning effect during contraction. The longitudinal muscle fibers are positive for phalloidin and anti-actin immunohistochemistry (Figure 7), and asymmetrically arranged around the periphery of the stalk in all four species. The muscle fibers splay out into the subumbrella from the rhopalial stalk when labeled with anti-actin (Figure 8).

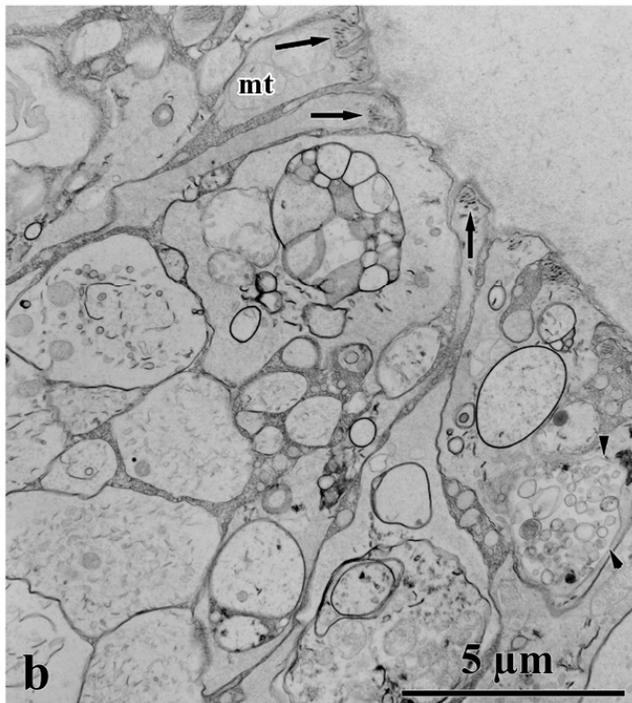
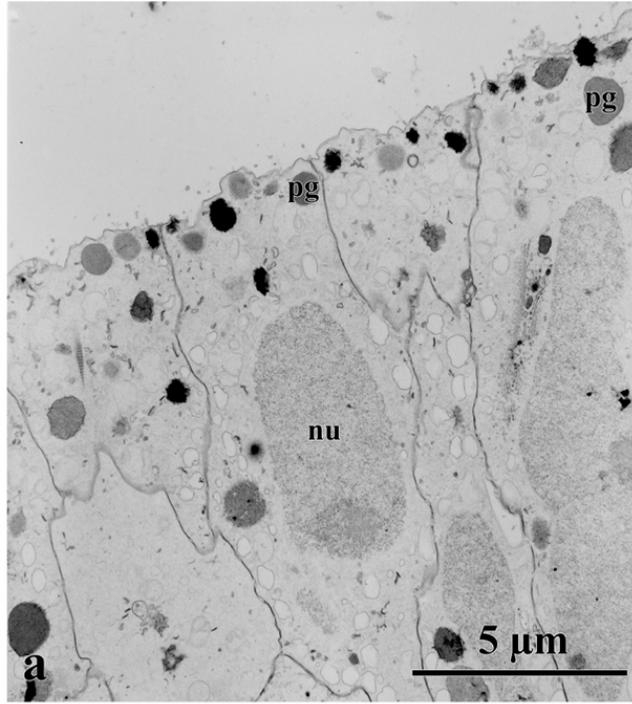
**Figure 3.** Organization of the rhopalial stalk of the box jellyfish. (a). A longitudinal, thick plastic section through the stalk and rhopalium of *Tripedalia cystophora* illustrating the gastrovascular cavity (GVC), the gastrodermis (gast), a small layer of mesoglea (arrow) and a single layer of epithelial cells (epi). (b) A longitudinal histological section of the rhopalium and rhopalial stalk of *Chiropsalmus quadrumanus*, stained with Gabe's trichrome. The single layer of epithelium (arrowheads) in the stalk stains red. The mesoglea (arrows) stains green, while the gastrodermis (asterisk) also appears red. The small complex eye (SCE) can be seen in the upper middle section of the micrograph and the large complex eye (LCE) in the bottom right.



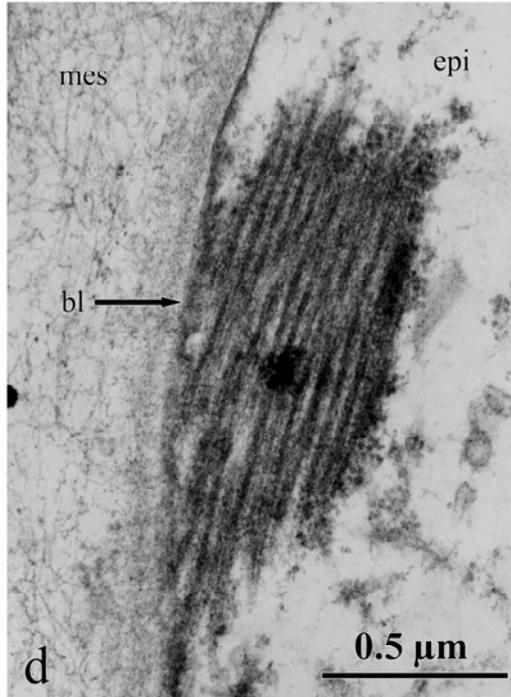
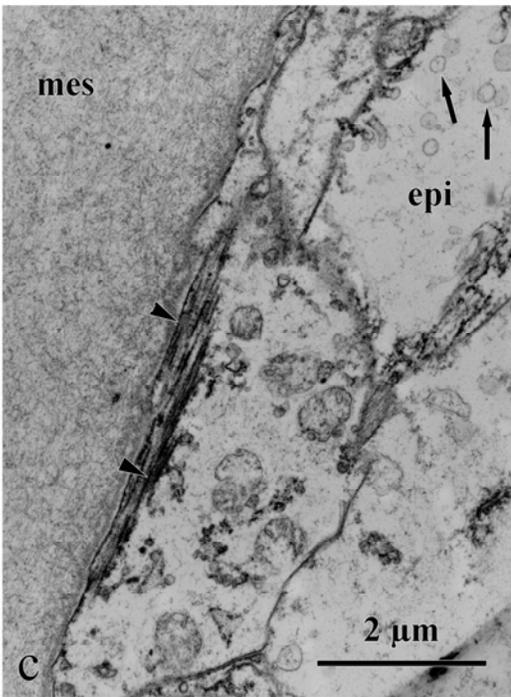
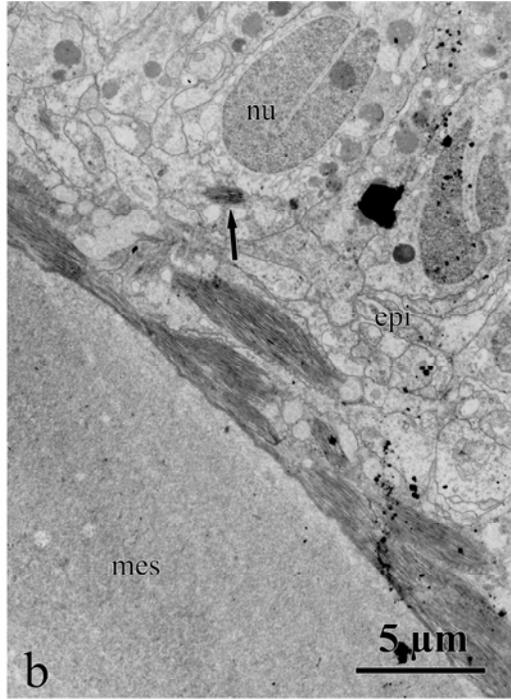
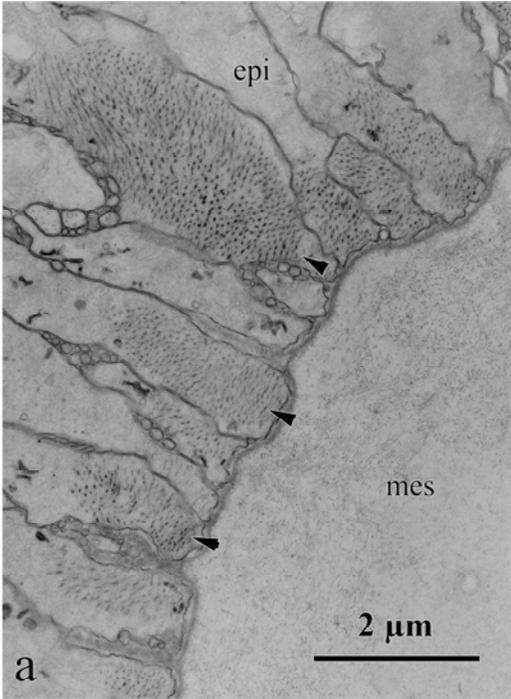
**Figure 4.** Micrograph of the epithelial junctions of the box jellyfish, shown here in *Chiropsalmus quadrumanus* (a). (b) High magnification of the same section illustrating the structure of the desmosomes (arrows) and septate junctions (arrowheads). Muscle fibers (mf) are grouped into myonemes at the basal end of the cells.



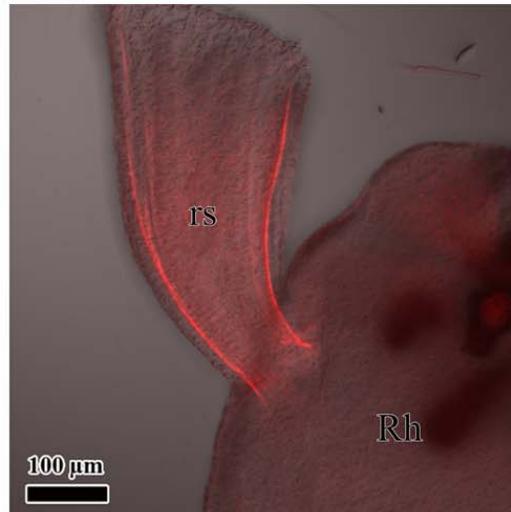
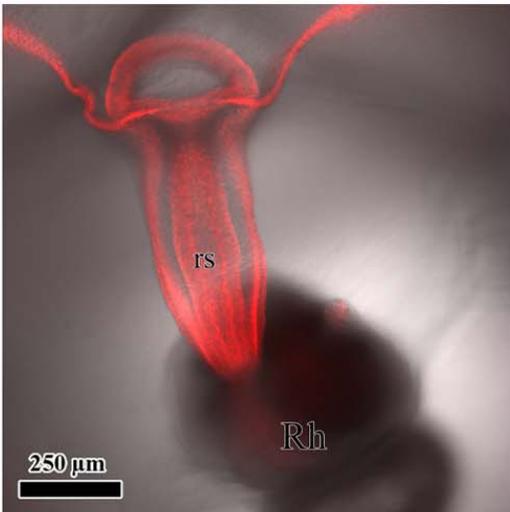
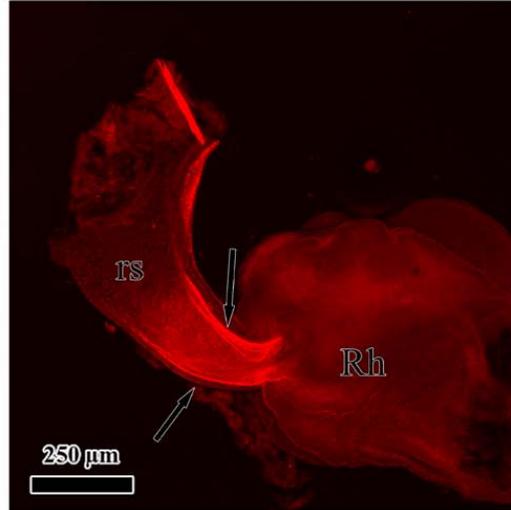
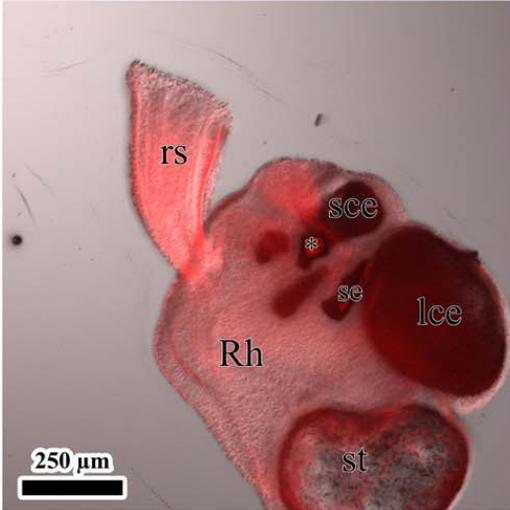
**Figure 5.** Epitheliomuscular cells of the stalk in the box jellyfish. (a) A single epitheliomuscular cell contains a nucleus (nu), pigment granules (pg) and microvilli at the apical end of the cell. (b) Muscle fibers (arrows) are found at the basal end of the cells. Mitochondria (mt) and clear synaptic vesicles (arrowheads) are usually seen around the epidermal nerve in the rhopalial stalk.



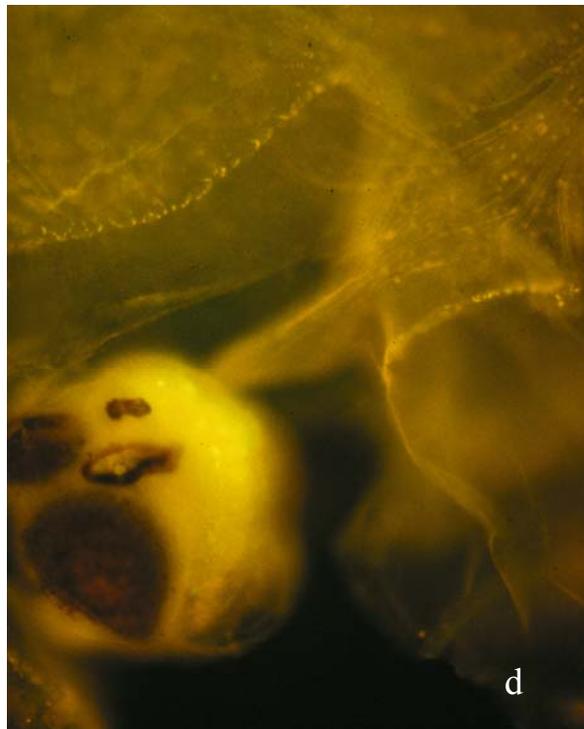
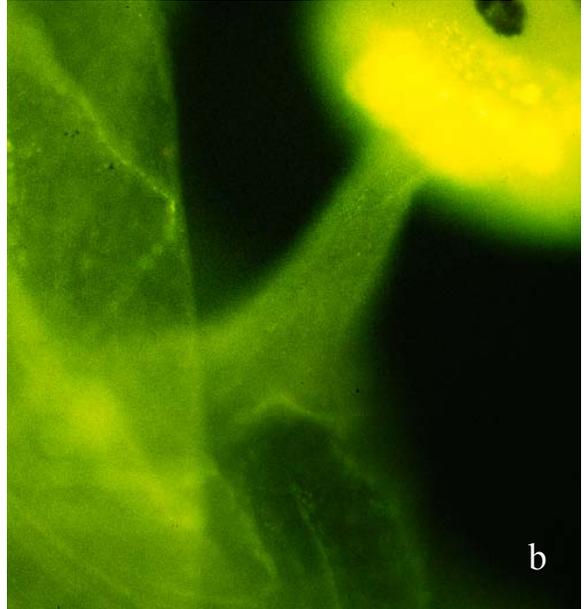
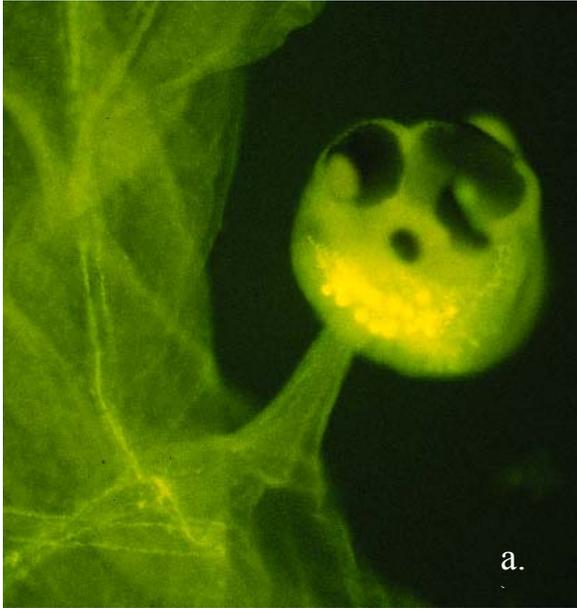
**Figure 6.** Muscle fibers in the rhopalial stalk of *Chiropsalmus quadrumanus*. (a) Cross-section of longitudinal muscle fibers (arrowheads) in the epithelium (epi) of the rhopalial stalk. (b) Longitudinal section of the rhopalial stalk illustrating the smooth, longitudinal muscle fibers in epitheliomuscular cells. The arrow indicates a small bundle of muscle fibers. Nu-nucleus. (c) High magnification of longitudinal muscle fibers in the stalk. Clear synaptic vesicles (arrows) are often found in the epithelium (epi) with smooth muscle fibers (arrowheads) at the basal end of the cell. (d) High magnification micrograph of smooth muscle fibrils in the epithelium (epi). Note the basal lamina (bl) and the absence of helical and circular muscle fibrils. epi – epidermis; mes-mesoglea.



**Figure 7.** Longitudinal muscle fibers in the stalk are positive for phalloidin. Shown here are different orientations of the rhopalial stalk (rs) labeled with phalloidin. (a) Each rhopalium (Rh) consists of sensory epithelia, a small complex eye (SCE), a large complex eye (LCE), two slit ocelli and two pit ocelli (\*), and a statolith (st). Only one slit eye (se) and one pit eye (\*) are seen here. (b) The rhopalial stalk (rs) and the rhopalium (Rh) from the aboral end, with the lateral side nearest the bottom of the micrograph. Arrows indicated bands of muscle fibers labeled with phalloidin. (c) Medial view of the rhopalium (Rh) suspended from the rhopalial stalk (rs) in the rhopalial niche. (d). High magnification of the rhopalial stalk (rs) and the attachment of the rhopalium (Rh).



**Figure 8.** Immunohistochemistry of the rhopalial stalk of *Tripedalia cystophora*. (a) Low magnification of the rhopalial stalk labeled with FMRF-amide. (b) High magnification of the same stalk in (a) labeled with FMRF-amide. (c) The connection of the stalk to the ring nerve, labeled here with tubulin. (d) Medial view of the rhopalium and the rhopalial stalk, illustrating the muscle in the stalk that splays out into the subumbrella.

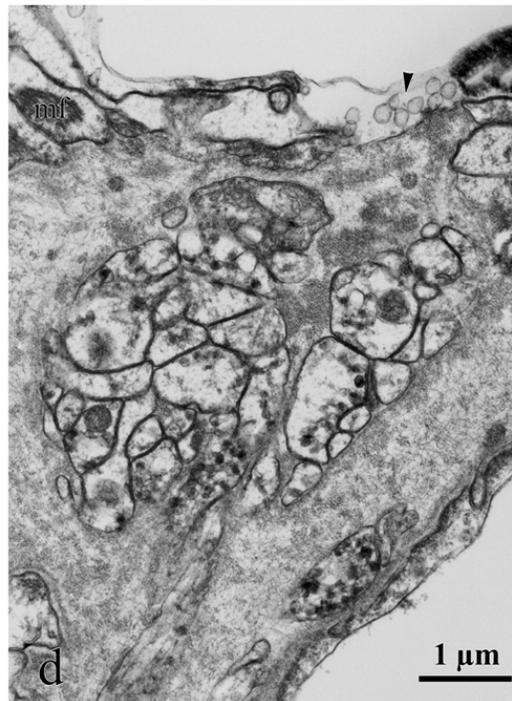
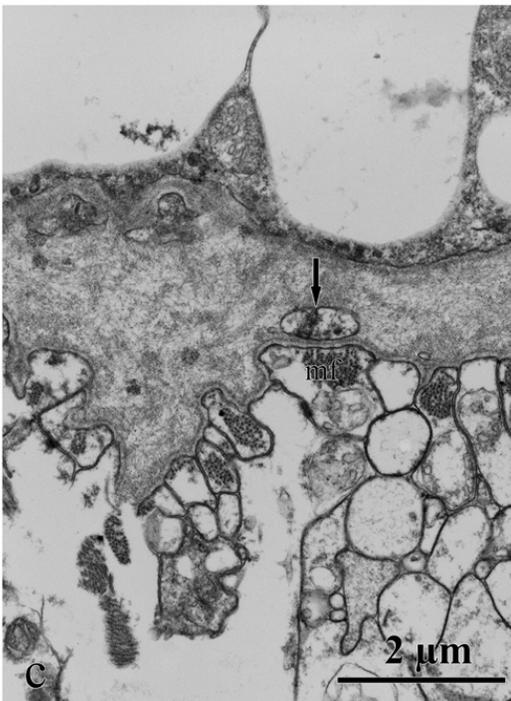
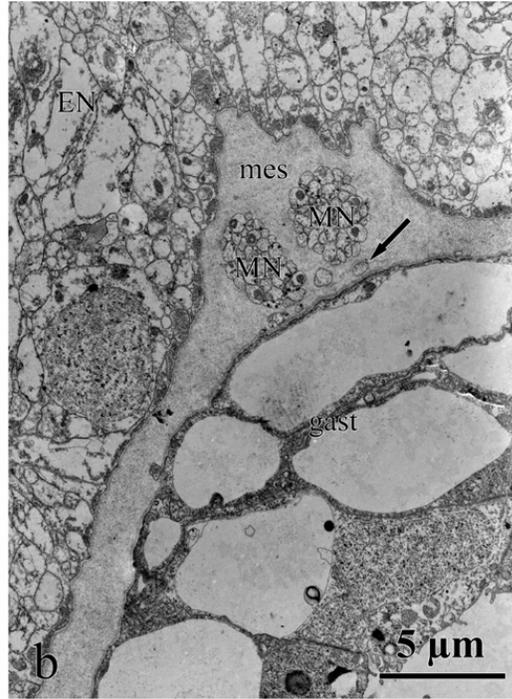
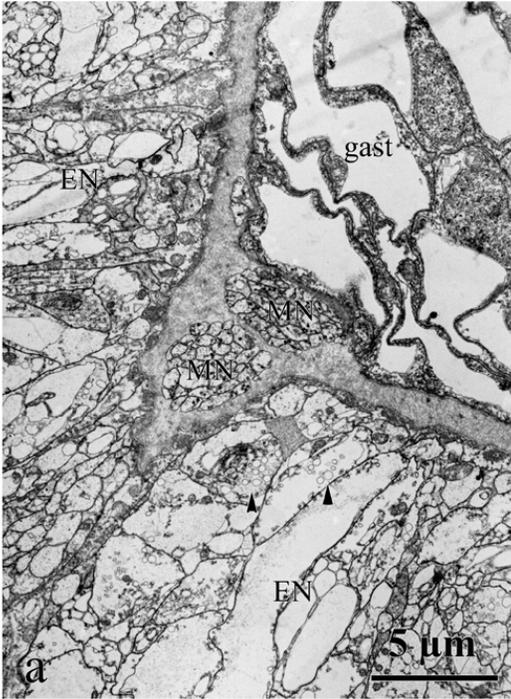


The epidermis thickens on the central (medial) side due to the presence of an epidermal nerve (EN) (Figures 1, 9). The EN is bi-lobed and consists of parallel nerve bundles traveling the full length of the stalk and ultimately ending inside the rhopalium where the neurites spread out. The neurites could not be followed at length inside the rhopalium. In the roof of the rhopalial niche, the EN synapses onto the ring nerve (Figure 8c). Both clear and dense-cored vesicles were found in and around the EN. The neurite cell bodies within the EN are void of organelles and consist mostly of cytoplasm, microtubules and muscle fibers, though the latter is not consistent through the length of the stalk in and around the EN.

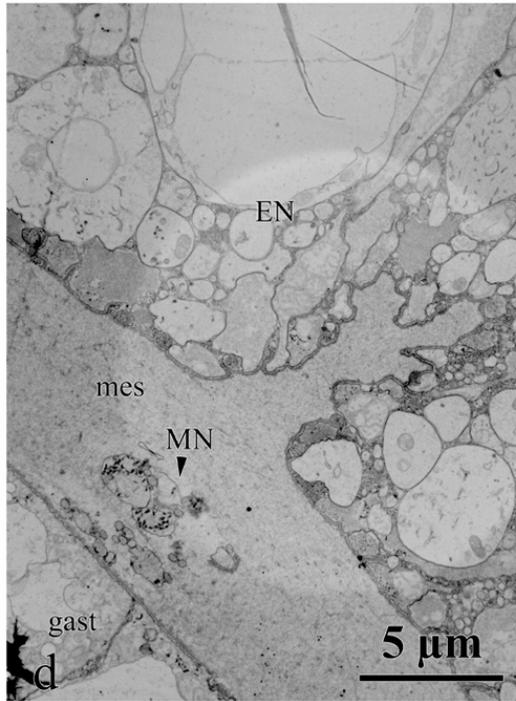
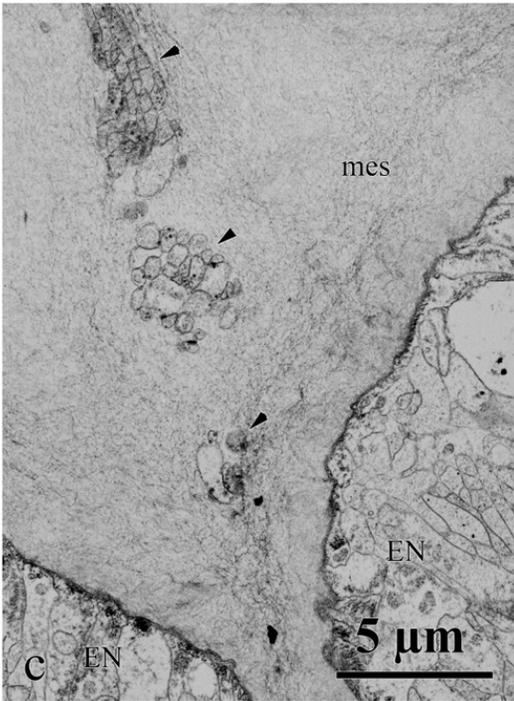
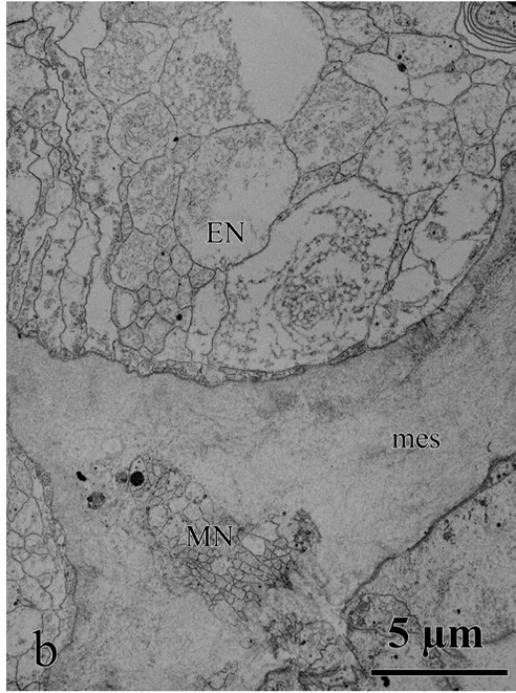
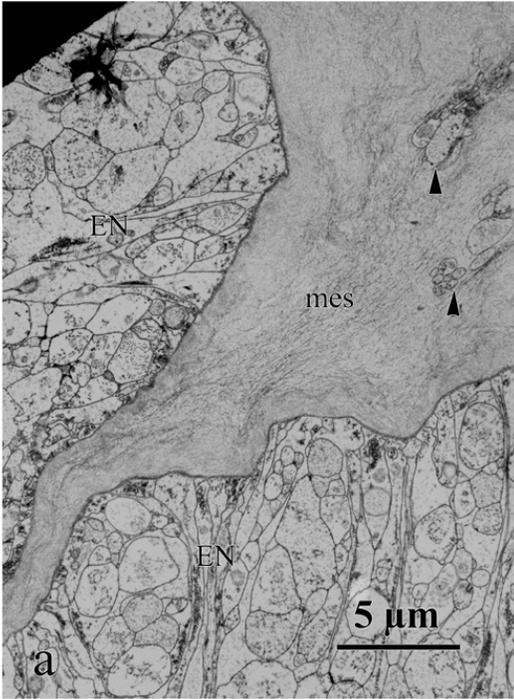
The mesoglea is a collagenous matrix that separates the endoderm and ectoderm (Figure 1). It decreases or increases in thickness corresponding to the thickness of the epidermis on the lateral side. A mesogleal nerve (MN) (Figures 9, 10), runs the majority of the length of the stalk in three out of the four species. It is normally found at an invagination of the epidermis on the medial side in the same general area as the EN. The MN is only found in *Tripedalia cystophora*, *Carybdea alata* and *C. marsupialis*. It was not found in any individual of the species *Chiropsalmus quadrumanus*. Additionally, the MN was not found in every individual of the three identified species (Figure 11).

The MN begins aborally but tapers off near the rhopalium, so it is present in the initial  $\frac{3}{4}$  of the stalk. The nerve is not always constant in structure throughout its length (Figure 9). It comes into close contact with both the epidermis and gastrodermis but always remains separate via a basement membrane. Dense-cored vesicles and mitochondria are found throughout the MN, and neurites increase in number, becoming smaller as the MN gradually tapers off. The neurites could not be followed as the MN gradually disappears, but nerve processes may run between both the gastrodermis and the epidermis. In *T. cystophora*, the nerve sometimes appears

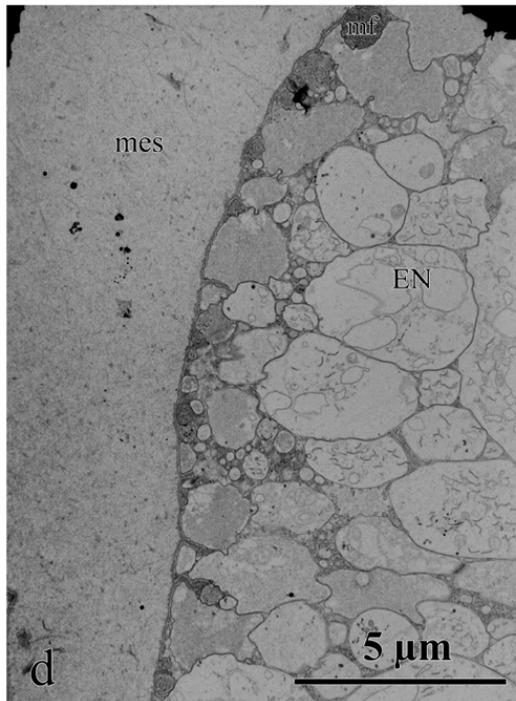
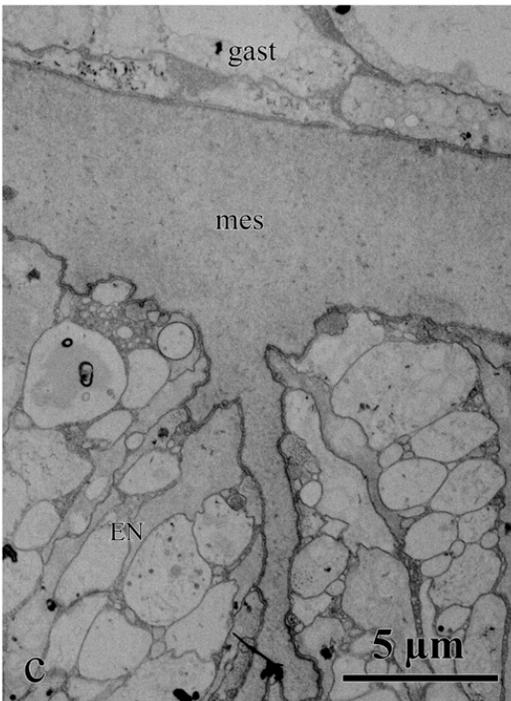
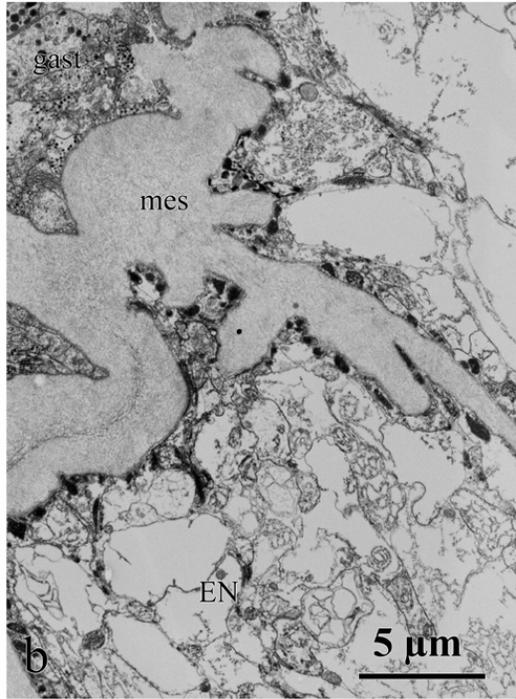
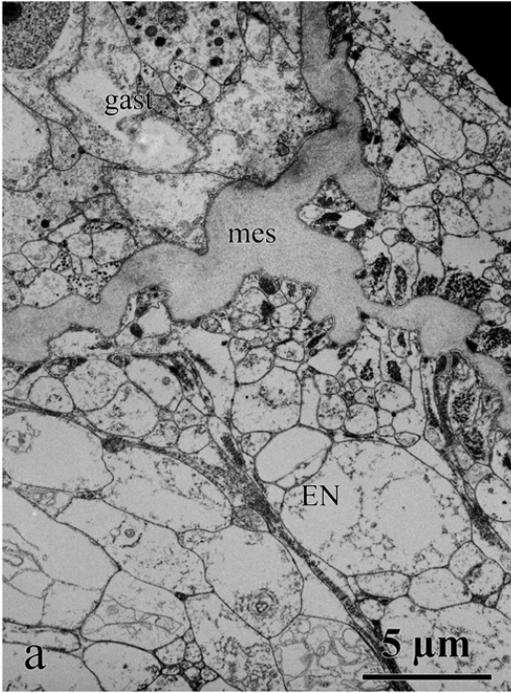
**Figure 9.** The epidermal and mesogleal nerve of the box jellyfish as it appears in *Tripedalia cystophora*. The mesogleal nerve (mn) appears branched in *Tripedalia cystophora* (a, b). Clear synaptic vesicles (arrowheads) are found in the epidermis, in the vicinity of the epidermal nerve (EN) (a,d). Occasionally, parts of the mesogleal nerve appear separated from the neurite bundle (arrow, b,c) as the nerve begins to fragment in the oral region of the stalk. High magnification of the mesogleal nerve (c, d). Bundles of muscle fibers (mf) are found in the epithelium around the periphery of the stalk.



**Figure 10.** The variability of the mesogleal nerve in the box jellyfish, shown here in *Carybdea marsupialis* (a, b, c) and *Carybdea alata* (d). The mesogleal nerve (mn) is found in the mesoglea (mes) at the invagination of the epithelium at the epidermal nerve (EN). It is variable in nature (a, arrowheads) and often appears segmented into components (c, arrowheads) throughout the majority of the stalk. Gast-gastrodermis.



**Figure 11.** The absence of the mesogleal nerve (MN) is notable in *Chiropsalmus quadrumanus* (a, b) and the oral region of *Carybdea alata* (c,d). The nerve was not seen in *Chiropsalmus quadrumanus* and tapers off in the latter ¼ of the stalk in the other three species. An invagination of the epidermis at the junction of the two lobes of epidermal nerve (EN) is present, but the absence of the MN is significant. Mes-mesoglea; gast- gastrodermis.



branched, and conjoined to the gastrodermis though it is in fact separated from it via a basement membrane (Figure 9 a,b). Neurite counts were not done for the MN in *Carybdea alata* and *C. marsupialis*, but in *T. cystophora* they appear to contain about 30 neurites in each node, or approximately 60 neurites at each cross-section.

The gastrodermis consists of cells that vary in structure, although most contain large vacuoles and those in close proximity to the gastrovascular cavity contain cilia. No muscle fibrils are found in the gastrodermis, which is homogenous throughout the length of the stalk.

### **Muscle fiber size**

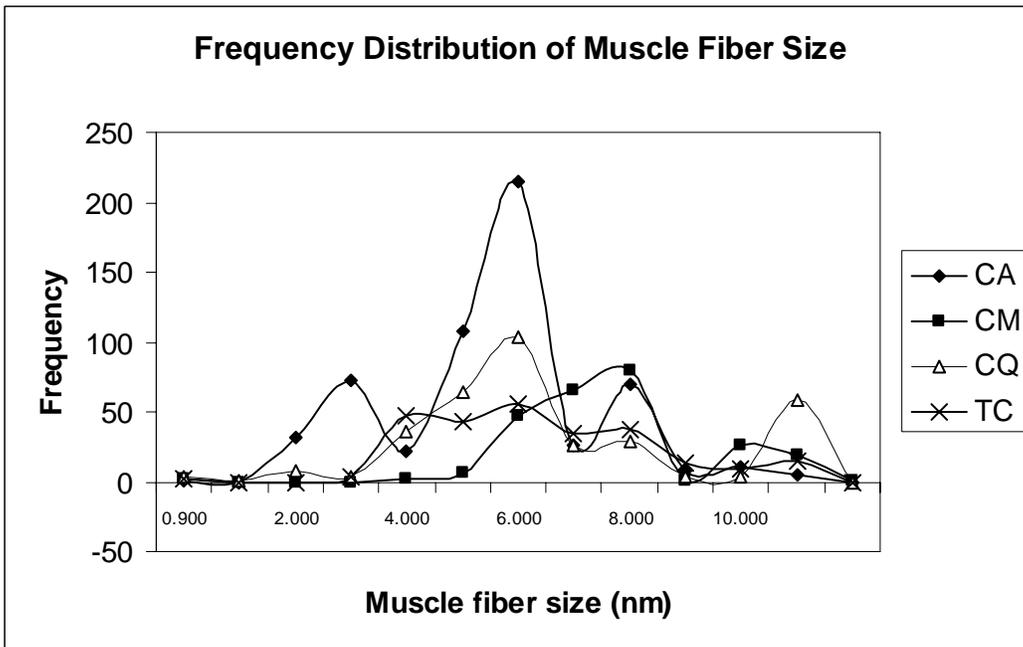
The cross-sectional area ( $\mu\text{m}^2$ ) of individual muscle fibers was measured for all four species in each region to determine if the diameter would change the length of the stalk, increasing, decreasing or no change. A frequency distribution of this size indicated that it is different for each species (Figure 12) and significant differences were found across species (MANOVA,  $F_2 = 113.204$ ,  $P < 0.05$ ) and regions (MANOVA,  $F_2 = 60.032$ ,  $P < 0.05$ ) of the stalk in *Carybdea alata*, *C. marsupialis* and *Chiropsalmus quadrumanus*. The results of the Tukey post-hoc test are found in table 3, illustrating that within *Carybdea alata*, *C. marsupialis* and *Chiropsalmus quadrumanus*, individual muscle fiber size significantly decreased the length of the stalk from the aboral to the oral region (Figure 12b). In *Carybdea alata*, the fiber size from the aboral to the middle region slightly increased, but the difference was not significant. *Carybdea marsupialis* generally had the largest individual muscle fiber size, while *Carybdea alata* had the smallest fiber size.

The size of individual fibers in *Tripedalia cystophora* was analyzed separately due to the absence of the aboral region where a significant difference was found between the middle and

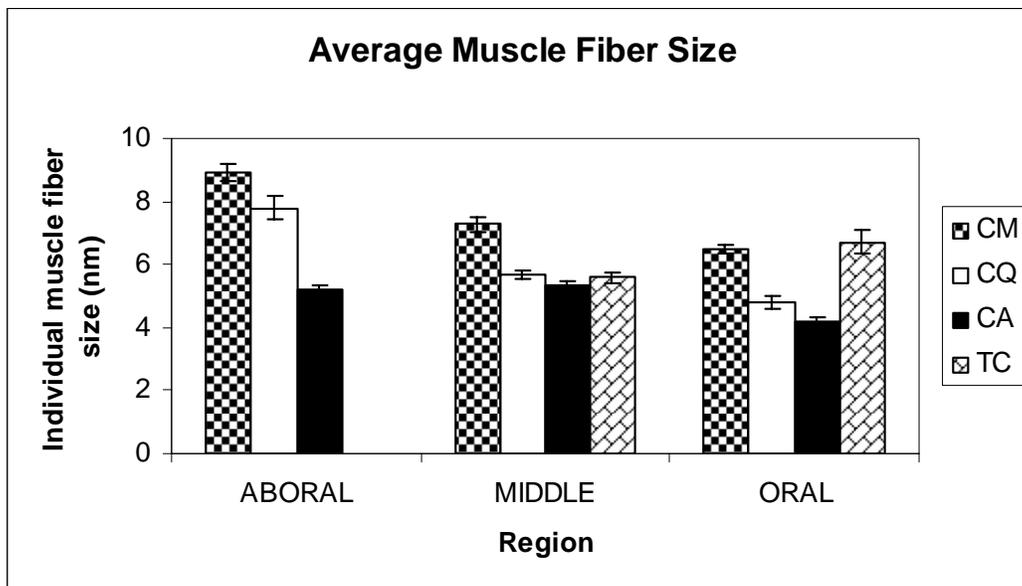
**Figure 12.** Frequency distribution and histogram (average nm  $\pm$  SEM) for individual muscle fiber size in cross-section for *Carybdea alata*, *Carybdea marsupialis*, *Chiropsalmus quadrumanus* and *Tripedalia cystophora*.

a.) Frequency distribution of individual muscle fiber size for all four species. *Carybdea alata* (CA; diamond) has the only clear peak at approximately 5.6 nm. *Carybdea marsupialis* (CM; square) peaks near 8 nm, while *Chiropsalmus quadrumanus* (CQ; triangle) is closer to 20 nm. *Tripedalia cystophora* (TC; x) does not show a clear peak, but displays a rather steady relationship from 4 to 8 nm.

b.) Average individual muscle fiber size for *Carybdea marsupialis* (CM; checker pattern), *Chiropsalmus quadrumanus* (CQ; white), *Carybdea alata* (CA; black) and *Tripedalia cystophora* (TC; cross-hatch). The muscle fiber size of *Carybdea marsupialis* is generally largest, followed by *Chiropsalmus quadrumanus*, and *Carybdea alata*. The general trend is a decreasing of fiber diameter from the aboral to the oral region. *T. cystophora* does not follow this trend, instead significantly increasing in size from the middle to the oral region. The aboral region of *T. cystophora* is not shown due to the absence of samples.



a.)



b.)

**Table 3.** Results of the Tukey-post hoc test for individual muscle fiber size ( $\mu\text{m}$ ) across species and regions for *Carybdea alata* (CA), *Carybdea marsupialis* (CM) and *Chiropsalmus quadrumanus* (CQ). ND (no difference) indicates no significant difference, while the given P-value indicates a significant difference ( $P = 0.05$ ,  $\text{DOF} = 2$ ).

		<i>C. alata</i>			<i>C. marsupialis</i>			<i>C. quadrumanus</i>		
		ABORAL	MIDDLE	ORAL	ABORAL	MIDDLE	ORAL	ABORAL	MIDDLE	ORAL
<b>CA</b>	<b>ABORAL</b>		ND	0.000621	0.000010	0.000010	0.000033	0.000010	ND	ND
	<b>MIDDLE</b>	ND		0.000202	0.000010	0.000010	0.002016	0.000010	ND	ND
	<b>ORAL</b>	0.000621	0.000202		0.000010	0.000010	0.000010	0.000010	0.000011	ND
<b>CM</b>	<b>ABORAL</b>	0.000010	0.000010	0.000010		0.000894	0.000010	0.047703	0.000010	0.000010
	<b>MIDDLE</b>	0.000010	0.000010	0.000010	0.000894		ND	ND	0.000011	0.000010
	<b>ORAL</b>	0.000033	0.002016	0.000010	0.000010	ND		0.000320	ND	0.001360
<b>CQ</b>	<b>ABORAL</b>	0.000010	0.000010	0.000010	0.047703	ND	0.000320		0.000010	0.000010
	<b>MIDDLE</b>	ND	ND	0.000011	0.000010	0.000011	ND	0.000010		ND
	<b>ORAL</b>	ND	ND	ND	0.000010	0.000010	0.001360	0.000010	ND	

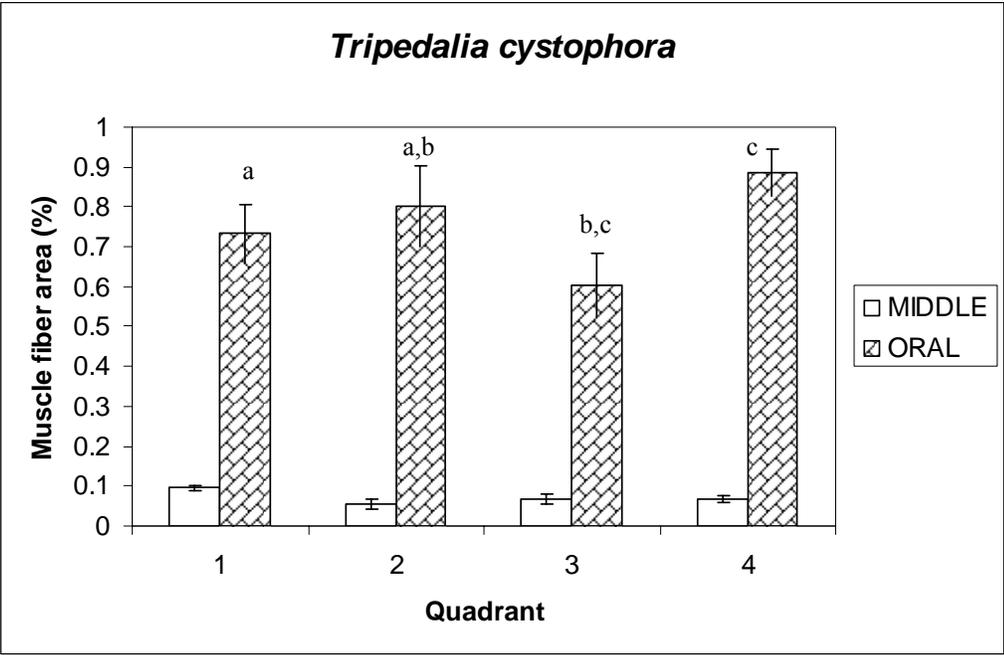
oral region (ANOVA,  $F_1 = 10.803$ ,  $P < 0.05$ ). Muscle fiber size in *T. cystophora* for the middle region was significantly lower than the size of the fibers in the oral region. Although *Tripedalia* was analyzed separately, all four species are displayed together in the same frequency distribution and histogram comparing the average individual muscle fiber size (Figure 12).

### **Muscle fiber distribution**

Transmission electron microscopy was used to analyze muscle fiber area per quadrant and per total stalk area. The area of muscle fibers per sample was analyzed per quadrant area and stalk area. The main difference between the two analyses was a significant difference between quadrant 1 and quadrant 2 analyzing percent muscle per quadrant across species (MANOVA,  $F_2 = 2.880$ ,  $P < 0.05$ ). Other than that small difference, no differences were found between the two analyses. Therefore, muscle fiber area per quadrant area will be the main focus. No significant difference was found between regions or quadrants, but rather between species (MANOVA,  $F_2 = 10.646$ ,  $P < 0.05$ ), regions and species (MANOVA,  $F_4 = 18.381$ ,  $P < 0.05$ ), and quadrants and species (MANOVA,  $F_6 = 2.156$ ,  $P < 0.05$ ). *Carybdea alata* was significantly different from both *Carybdea marsupialis* and *Chiropsalmus quadrumanus*, although no difference was found between the latter species.

*Tripedalia cystophora*. *T. cystophora* density was analyzed separately from that of the other three species due to the absence of samples from the aboral region. The oral region contained a significantly higher percentage of muscle fiber area than the middle region (ANOVA,  $F_1 = 217.659$ ,  $P < 0.05$ ). The results of the Tukey-HSD test for quadrants are illustrated in Figure 13 where the same letter above each respective column in the oral region indicates significant differences. No significant differences were found among quadrants in the

**Figure 13.** Muscle fiber percent area ( $\% \pm \text{SEM}$ ) for *Tripedalia cystophora* in the middle and oral regions. The aboral region was not sampled. The middle (white) and oral (cross-hatch) regions were significantly different from each other. Within the middle region, no quadrants were found to be significantly different. However, in the oral region, significant differences were found between quadrants 1 and 2, 2 and 3, and 3 and 4. The same letters above the columns in the oral region indicate significant differences between those quadrants.



middle region. In the oral region, there was a significant difference between quadrants 1 and 2, 2 and 3, and 3 and 4. Percentage of muscle fiber area is greater in quadrants 2 and 4.

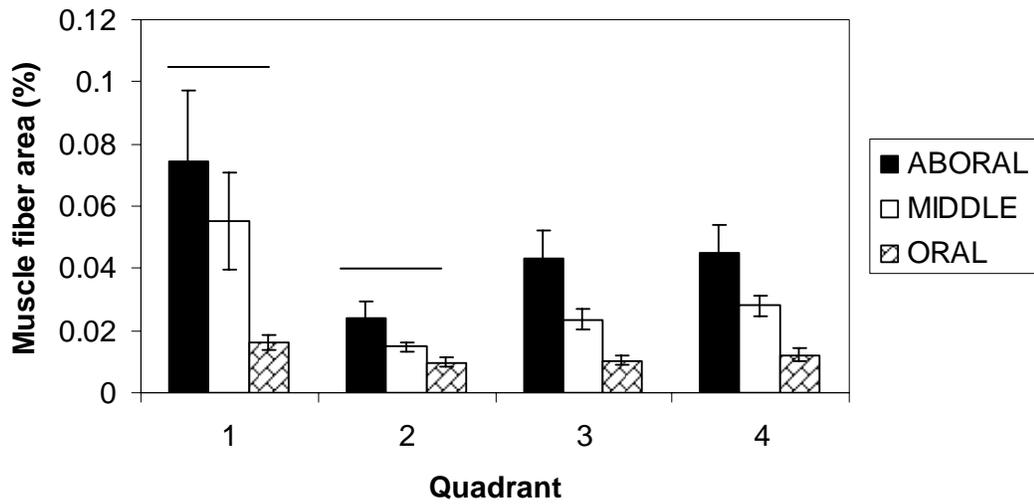
*Chiropsalmus quadrumanus*. The oral region of *C. quadrumanus* had significantly less percent area of muscle fibers than the aboral region (Figure 14). A larger percentage of muscle fiber area is also found between the quadrants although the differences were not significant. Looking at the complete length of the stalk, quadrant 1 contains a significantly greater percent area of muscle fibers than quadrant 2. While it appears that the aboral and middle regions in quadrant 1 have a larger percent of muscle fibers than the other three quadrants, no significant difference was found.

*Carybdea alata*. Within the stalk of *C. alata*, the oral region was significantly different from both the aboral and middle regions with a greater percent area of muscle fibers (Figure 15). Quadrant 1 had significantly less percent area of muscle fibers than quadrant 2 throughout the length of the stalk. Therefore, quadrants 2, 3, and 4 contain approximately the same percentage of muscle fibers.

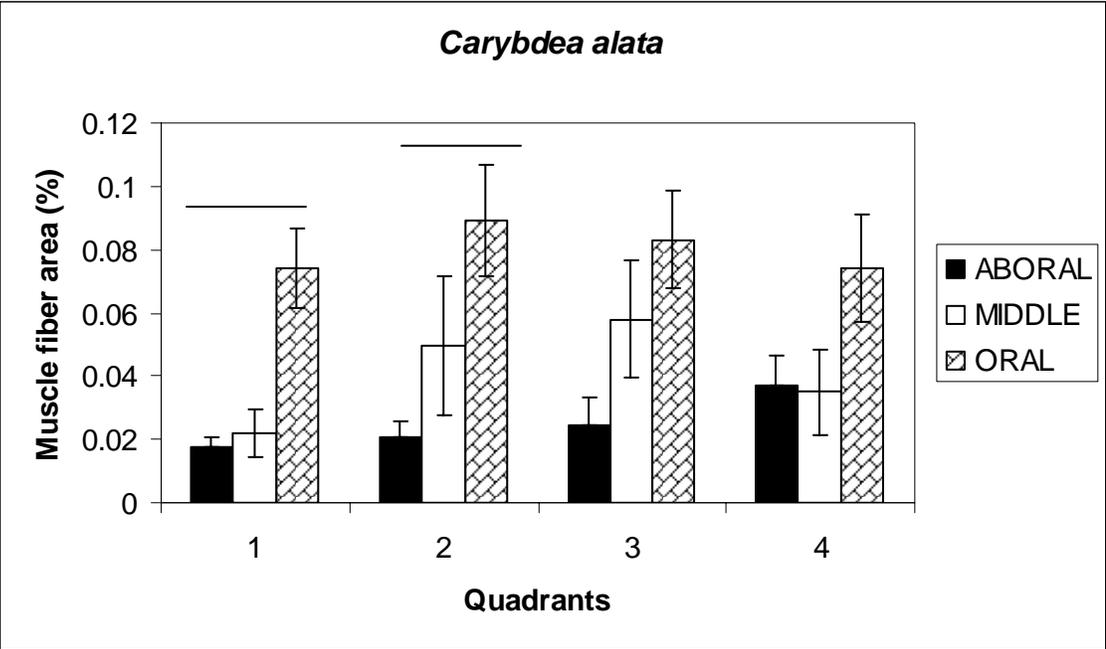
*Carybdea marsupialis*. In *C. marsupialis*, no regions were significantly different from each other. The only difference found was between quadrants 1 and 4 (Figure 16). Quadrant one had significantly more muscle fiber percent the length of the stalk than quadrant four. Inter-species relationships. Significant differences were found when comparing the regions against species (MANOVA,  $F_4 = 18.381$ ,  $P < 0.05$ ). The results of the Tukey post-hoc test are found in Table 4, where the given p-value represents significant difference between those two factors and no significant difference is indicated (ND). The oral region of *Carybdea alata* was significantly different from every other region in the analyses (Figure 17) with a higher percentage of muscle fiber area than any other region. The aboral region of *Chiropsalmus*

**Figure 14.** Muscle fiber percent area ( $\% \pm \text{SEM}$ ) in *Chiropsalmus quadrumanus*. Muscle fiber percent is shown for the aboral (black), middle (white) and oral (crosshatched) regions. The bars above quadrants 1 and 2 indicate a significant difference between those quadrants. No other significant differences were found.

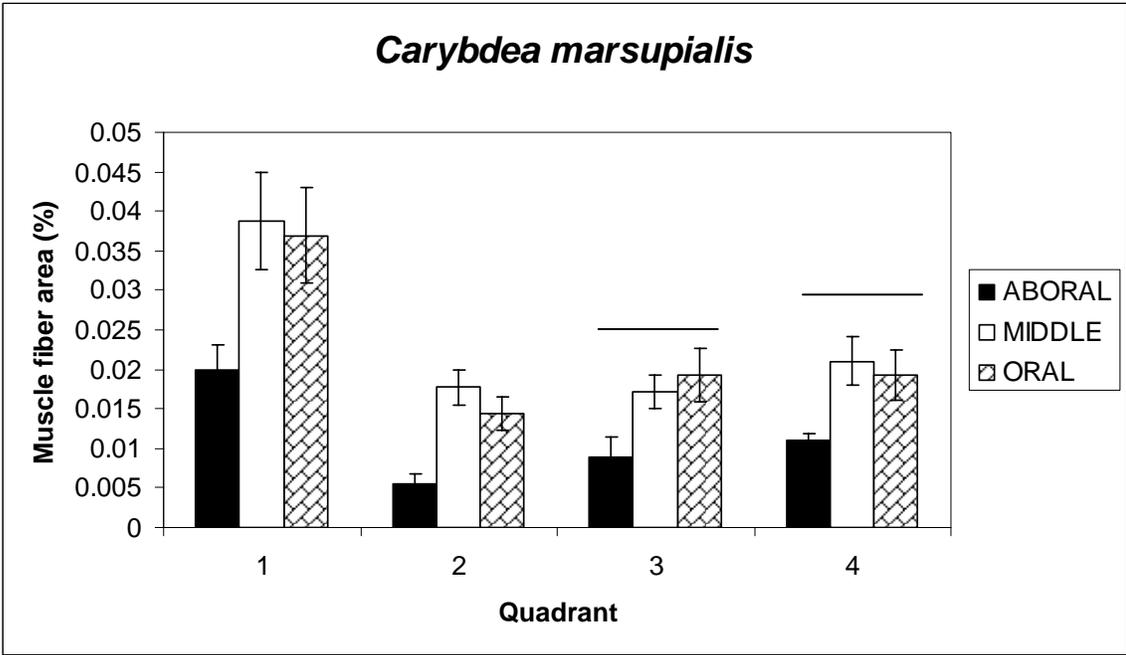
*Chiropsalmus quadrumanus*



**Figure 15.** Muscle fiber percent ( $\% \pm \text{SEM}$ ) in *Carybdea alata*. Percent area is shown for the aboral (black), middle (white) and oral (cross-hatched) regions. A bar above the columns for quadrants 1 and 2 indicate a significant difference. No other significant differences were found.



**Figure 16.** Percentage of muscle fiber area (% muscle fiber area  $\pm$  SEM) for *Carybdea marsupialis*. Aboral (black), middle (white) and oral (cross-hatched) regions are represented. The bars above quadrants 3 and 4 indicate a significant difference between those two quadrants. No other significant differences were found.

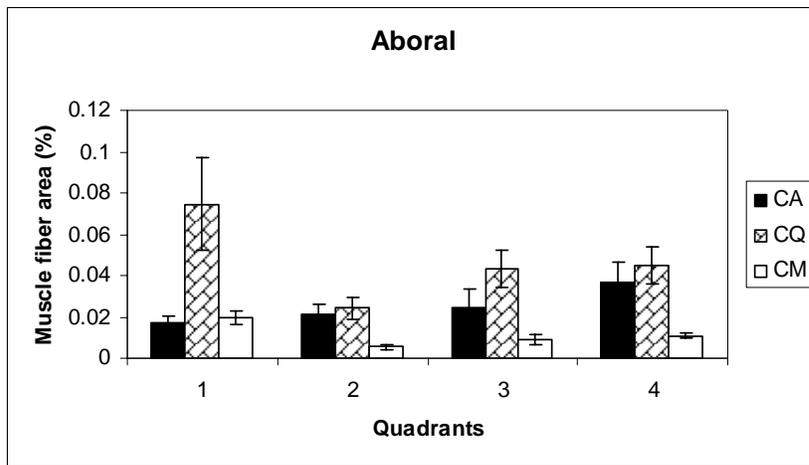


**Table 4.** Results of the Tukey-HSD post-hoc test for percent area of muscle fibers across regions for *Carybdea alata* (CA), *Chiropsalmus quadrumanus* (CQ) and *Carybdea marsupialis* (CM). Significant differences are indicated by the given P-values (P=0.05, DOF=4). No significant difference is indicated by ND (no difference).

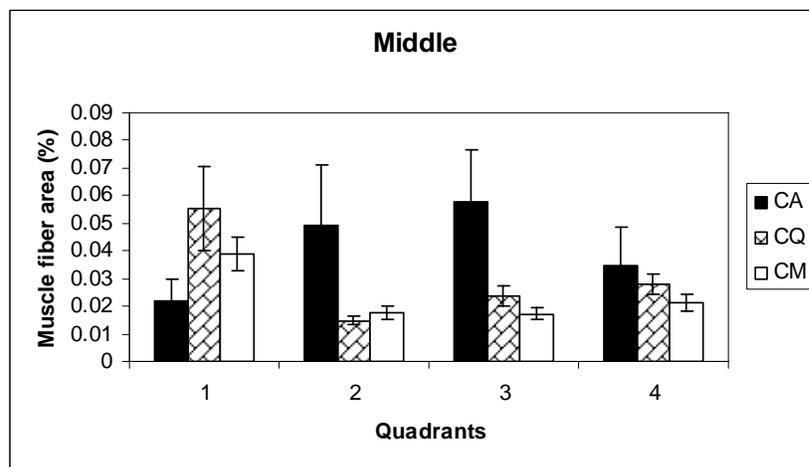
		ABORAL			MIDDLE			ORAL		
		CA	CQ	CM	CA	CQ	CM	CA	CQ	CM
ABORAL	CA		ND	ND	ND	ND	ND	0.00001	ND	ND
	CQ	ND		0.01525	ND	ND	ND	0.00036	0.00019	ND
	CM	ND	0.01525		ND	ND	ND	0.00001	ND	ND
MIDDLE	CA	ND	ND	ND		ND	ND	0.00001	ND	ND
	CQ	ND	ND	ND	ND		ND	0.00001	0.04182	ND
	CM	ND	ND	ND	ND	ND		0.00001	ND	ND
ORAL	CA	0.000011	0.00036	0.000010	0.00001	0.00001	0.00001		0.00001	0.00001
	CQ	ND	0.00019	ND	ND	0.04181	ND	0.00001		ND
	CM	ND	ND	ND	ND	ND	ND	0.00001	ND	

**Figure 17.** Comparison of percentage of muscle fiber area ( $\% \pm \text{SEM}$ ) for *Carybdea alata* (CA, black), *Chiropsalmus quadrumanus* (CQ, cross-hatch) and *Carybdea marsupialis* (CM, white). *Tripedalia cystophora* was not included in the analyses as the aboral region was absent from this species. The oral region of *C. alata* (asterisk) was significantly different from all other regions in the Tukey (HSD) post-hoc test.

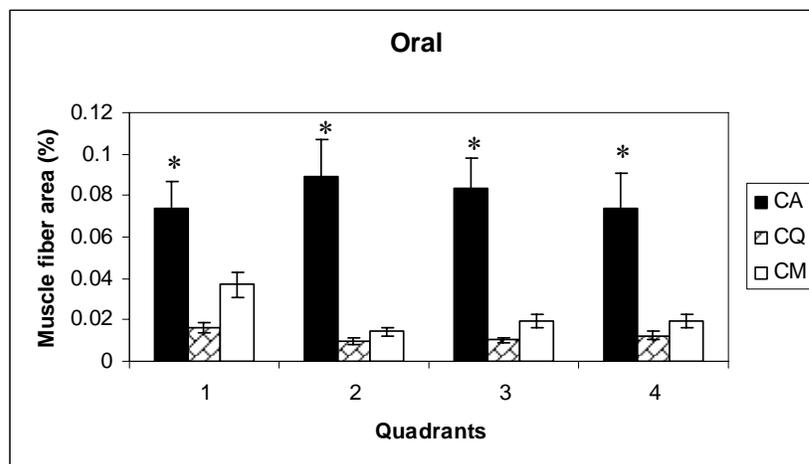
- a.) aboral
- b.) middle
- c.) oral



a.)



b.)



c.)

*quadrumanus* contained a significantly greater percentage of muscle fibers than the aboral region of *Carybdea marsupialis* and the oral region of *Chiropsalmus*. A significant difference was also found between the middle and oral region of *Chiropsalmus*, where the middle region contained a higher percent area of muscle fibers.

Many significant differences were found across quadrants and species (MANOVA,  $F_6 = 2.156$ ,  $P < 0.05$ ). The results of the Tukey post-hoc test are found in table 5. In a few instances, an increase in sample size may have affected the results such as between quadrant 3 of *Carybdea alata* and quadrant 3 of *Chiropsalmus quadrumanus* ( $P = 0.069$ ,  $DOF = 6$ ). Quadrant 3 of *Chiropsalmus* and quadrant 1 of *Carybdea marsupialis* were not found to be significantly different from any other quadrant within and among species. Most notable is that all four quadrants for *Carybdea alata* are different from quadrant 2 of *C. marsupialis*. Additionally, quadrants 2, 3, and 4 of *C. alata* are significantly different from quadrant 2 of *Chiropsalmus quadrumanus* and quadrant 1 was nearly significant ( $P = 0.079$ ,  $DOF = 6$ ). A lower muscle fiber percentage was found in quadrant 2 of both *Carybdea marsupialis* and *Chiropsalmus quadrumanus* when compared to other quadrants in each respective species, but was not found to be significant. A significant difference was only found in *C. quadrumanus*, between quadrants 1 and 2.

### **Vector analysis**

To better clarify the data, vector diagrams were completed for each species in each region to determine the potential direction of movement during contraction and to more accurately compare this putative movement across species and regions. Two assumptions were made while completing this analysis. First, it was assumed that all of the muscle fibers are symmetrically

**Table 5.** Results of the Tukey (HSD) post-hoc test for quadrants in *Carybdea alata*, *Chiropsalmus quadrumanus* and *Carybdea marsupialis* for muscle fiber percent area. No difference (ND) indicates no significant difference while P-values are shown for those that are significantly different (P = 0.05, DOF = 6).

		<i>C. alata</i>				<i>C. quadrumanus</i>				<i>C. marsupialis</i>			
		1	2	3	4	1	2	3	4	1	2	3	4
<i>C. alata</i>	1		ND	ND	ND	ND	ND	ND	ND	ND	0.04	ND	ND
	2	ND		ND	ND	ND	0.016	ND	ND	ND	0.0008	0.031	ND
	3	ND	ND		ND	ND	0.0008	ND	ND	ND	0.0004	0.002	0.008
	4	ND	ND	ND		ND	0.049	ND	ND	ND	0.027	ND	ND
<i>C. quadrumanus</i>	1	ND	ND	ND	ND		0.029	ND	ND	ND	0.015	ND	ND
	2	ND	0.016	0.0008	0.049	0.029		ND	DN	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND		ND	ND	ND	ND	ND
	4	ND	ND	ND	ND	ND	ND	ND		ND	ND	ND	ND
<i>C. marsupialis</i>	1	ND	ND	ND	ND	ND	ND	ND	ND		ND	ND	ND
	2	0.04	0.008	0.0004	0.027	0.015	ND	ND	ND	ND		ND	ND
	3	ND	0.031	0.002	ND	ND	ND	ND	ND	ND	ND		ND
	4	ND	ND	0.008	ND	ND	ND	ND	ND	ND	ND	ND	

activated at once, and not differentially activated by quadrant or region. Second, muscle fiber area was assumed to be related to contractile force, thus an increase in muscle density would indicate an increased contraction. In the vector diagrams, the direction of the arrow indicates the direction of summed bending, while the magnitude of the directional bending is indicated by the length of the arrow. The subtraction of the respective vector directions yields a rectangle, in which a directional arrow can be obtained. This arrow, and its length, indicates the strength of the contraction and in which direction the stalk would bend.

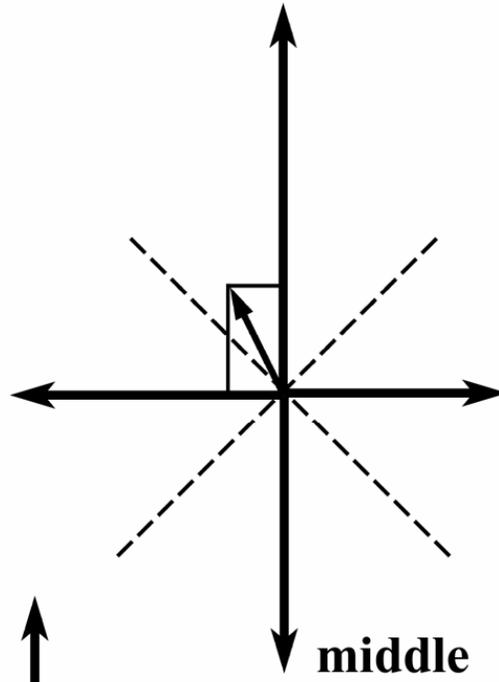
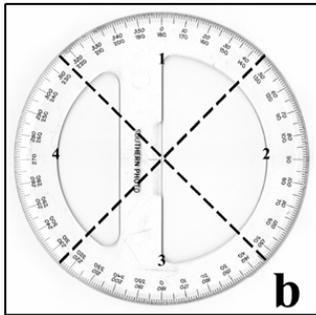
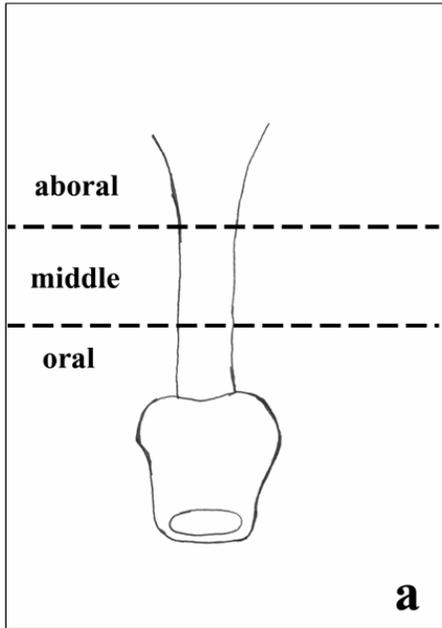
*Tripedalia cystophora.* In *Tripedalia cystophora*, the aboral region was not included due to the absence of samples (Figure 18). The direction of contraction is towards quadrant's 1 and 4 and is approximately the same from the middle region to the oral region, only changing in size. Note the difference in scale on the oral region here as the oral region had a significantly greater percent area of muscle fibers and had to be reduced in scale to more easily compare the two regions.

*Chiropsalmus quadrumanus.* The direction of contraction in *Chiropsalmus quadrumanus* changes from the aboral to the oral end, with the angle decreasing from the aboral to the oral end (Figure 19). As a result, in the aboral region the contraction would be more exaggerated towards quadrant 1, and in the oral region, more towards quadrant 4. The anatomical vector also indicates that the strength of the bending or shortening would also change, decreasing from the aboral to the oral end.

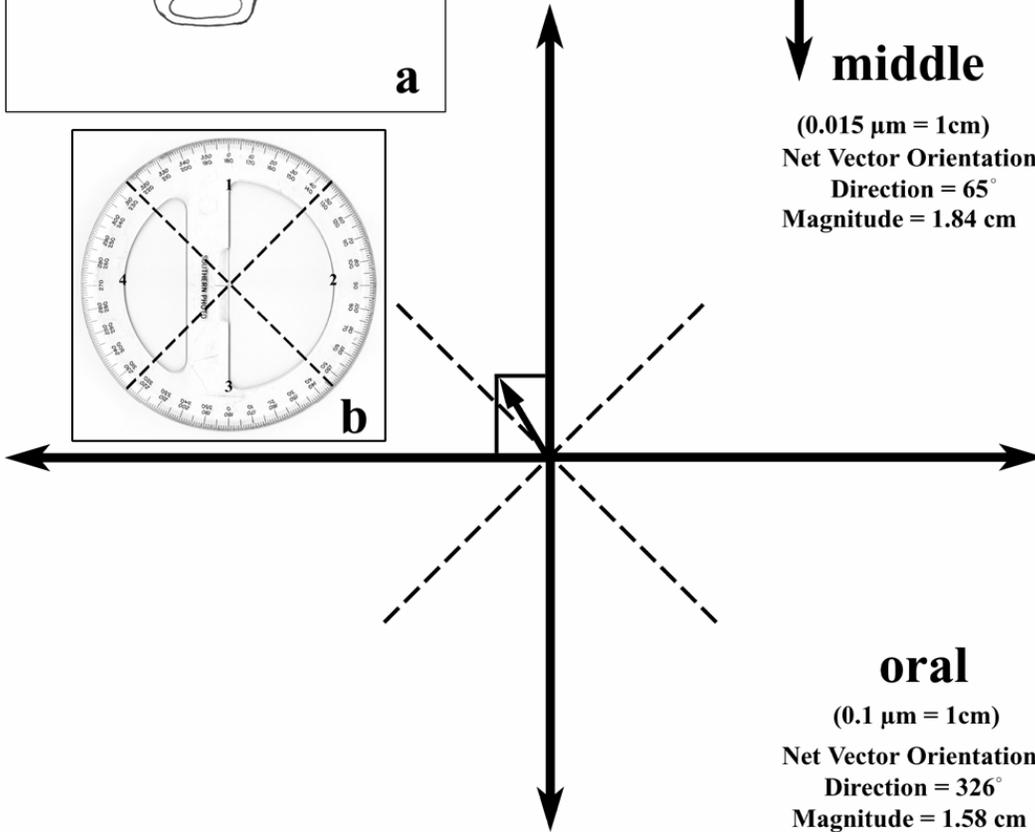
*Carybdea alata.* Within *Carybdea alata*, the length of the vector directions increases from the aboral to the oral end, with the oral region being significantly different from both the aboral and middle regions (Figure 20). The directional angle changes from the aboral to the oral

**Figure 18.** The vector diagram for *Tripedalia cystophora*. (a) The rhopalial stalk was divided into three regions. Shown here are the vector diagrams for the middle and oral region. The aboral region is not shown due to the absence of samples. The scale is different from the middle (b;  $0.015 \mu\text{m} = 1 \text{ cm}$ ) to the oral region (c;  $0.1 \mu\text{m} = 1 \text{ cm}$ ). (b) The protractor provides a reference for orientation. Direction of arrows designates the quadrant, while the length indicates the percent area of muscle fibers. Directional vectors were subtracted from each other to obtain the sum of vectors as indicated by the final vector angle. The net vector orientation is given in terms of final direction (orientation) for each region and the length of the resultant vector (magnitude).

*Tripedalia cystophora*



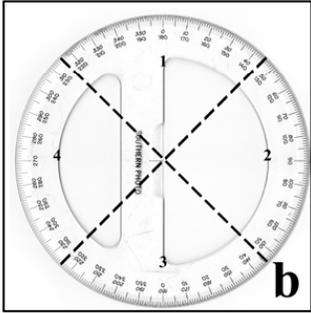
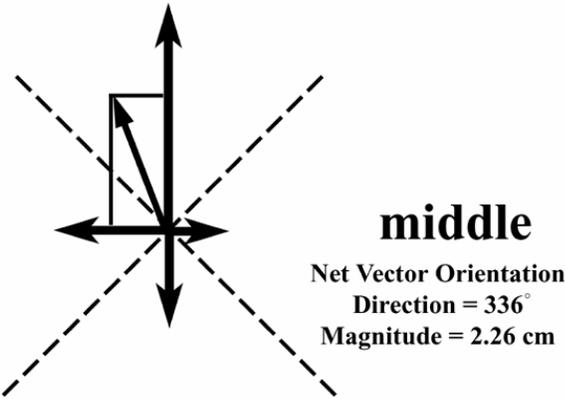
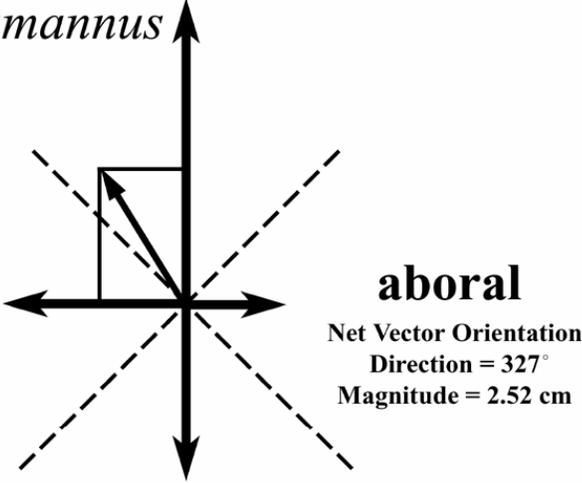
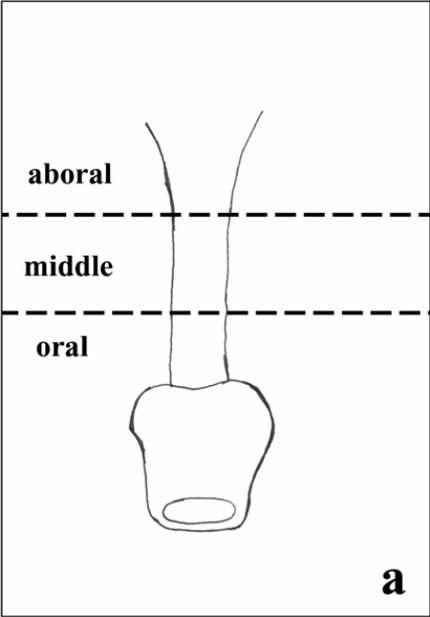
(0.015  $\mu\text{m}$  = 1cm)  
 Net Vector Orientation  
 Direction = 65°  
 Magnitude = 1.84 cm



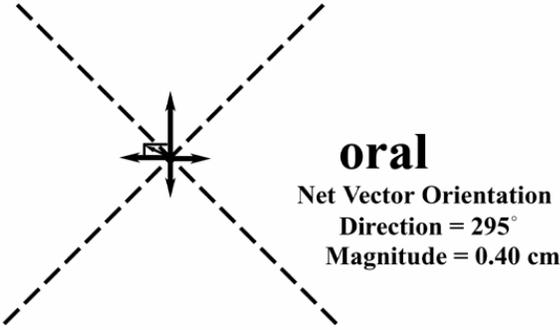
**oral**  
 (0.1  $\mu\text{m}$  = 1cm)  
 Net Vector Orientation  
 Direction = 326°  
 Magnitude = 1.58 cm

**Figure 19.** Vector diagram for *Chiropsalmus quadrumanus*. (a) The rhopalial stalk was divided into three regions: aboral, middle, and oral. (b) The protractor provides a reference for orientation. Direction of arrows designates the quadrant, while the length indicates percent area of muscle fibers ( $0.015 \mu\text{m} = 1 \text{ cm}$ ). Directional vectors were subtracted from each other to obtain a final vector angle. The net vector orientation is given in terms of direction (orientation) for each region and the length of the resultant vector (magnitude).

*Chiropsalmus quadrumannus*

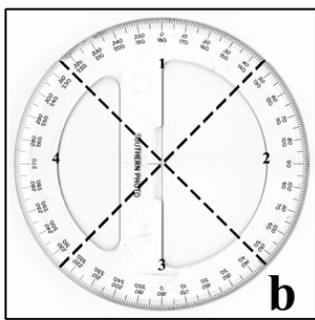
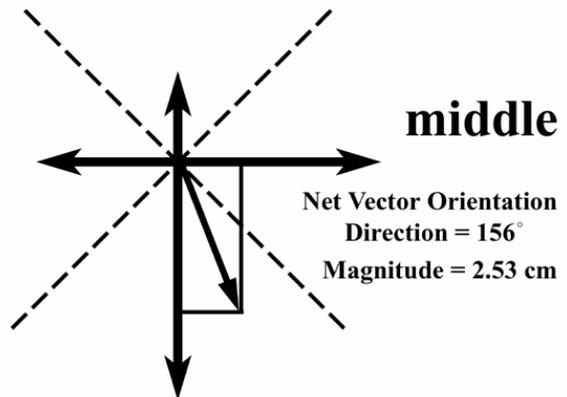
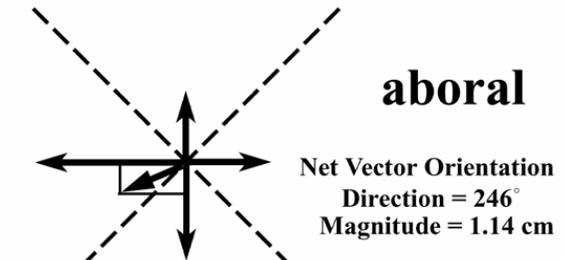
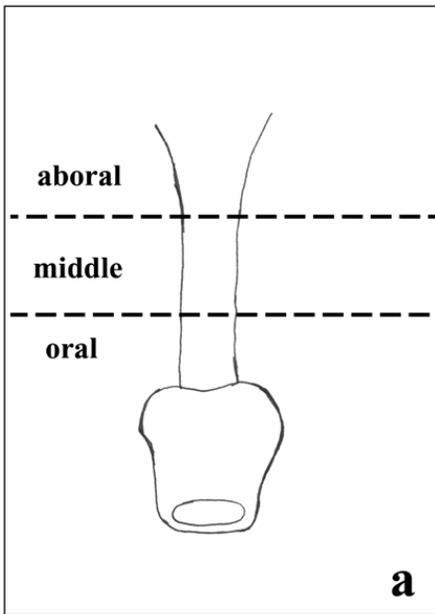


(0.015 μm = 1cm)

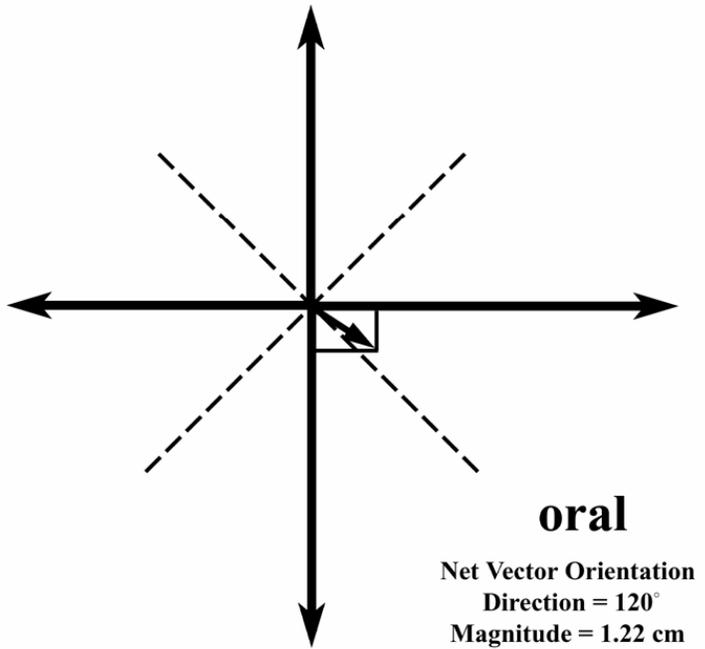


**Figure 20.** Vector diagram for *Carybdea alata*. (a) The rhopalial stalk was divided into three regions for which muscle fiber percent area was determined: the aboral, middle and oral. (b) The protractor provides a reference for orientation. Direction of arrows designates the quadrant, while the length indicates the percent area of muscle fiber ( $0.015 \mu\text{m} = 1 \text{ cm}$ ). Directional vectors were subtracted from each other to obtain a final vector angle as indicated by the net vector orientation. The final direction, orientation, is given for each region as well as the length of the resultant vector (magnitude).

*Carybdea alata*



(0.015  $\mu\text{m}$  = 1cm)



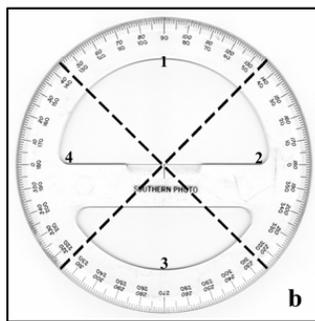
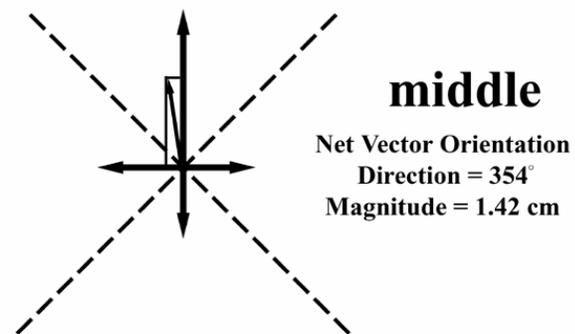
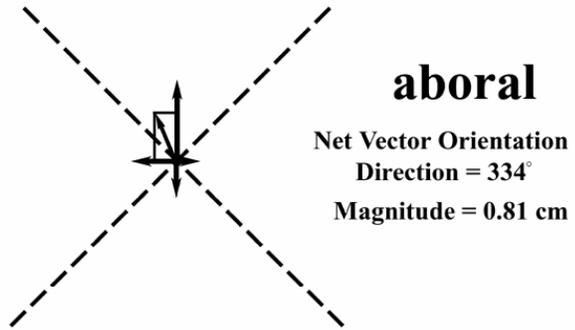
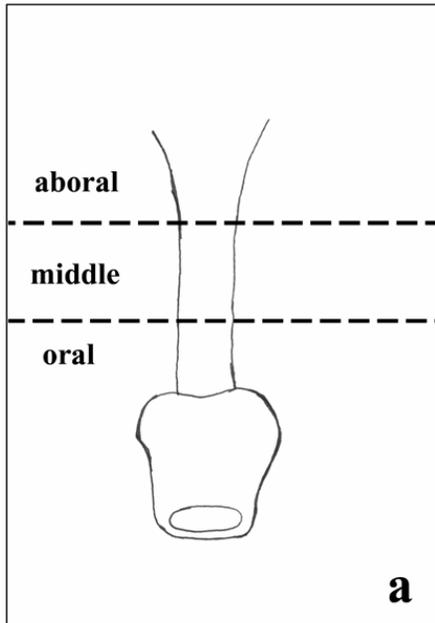
region as well. In aboral region, the direction is more towards quadrants 3 and 4, while in the middle and oral regions, it is closer to quadrants 2 and 3. The total directional bend would be towards quadrant 3, directly across from quadrant 1.

*Carybdea marsupialis*. The length of the vectors in *Carybdea marsupialis* generally increases moving from the aboral to the oral end, though not significantly (Figure 21). The direction of contraction in each region is towards quadrant 1, with a total directional bend in quadrant 1.

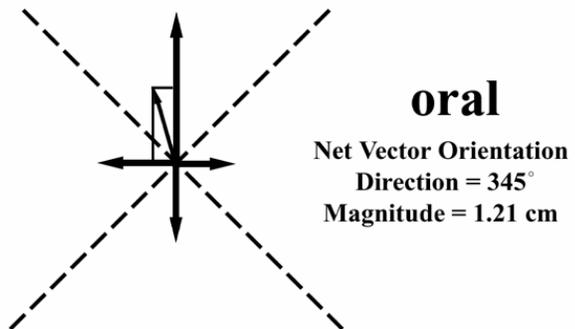
A summary diagram was constructed to illustrate the differences across species, where the length and direction of the resultant vector differ (Figure 22). In *Tripedalia cystophora*, *Chiropsalmus quadrumanus* and *Carybdea marsupialis*, the direction is generally towards quadrant 1 although the size of the vector differs in each region. However, in *Carybdea alata*, the resultant vector direction is near the center of quadrant 3, although in the aboral region the direction is more towards quadrants 3 and 4. In *Carybdea alata*, the vector length remains nearly the same. In *T. cystophora*, the vector length increases from the middle to the oral region by approximately five. In *Carybdea marsupialis*, the length of vectors gradually increases, from the aboral to the oral region. However, in *Chiropsalmus quadrumanus*, the size generally decreases from the aboral to the oral region, essentially the reverse of *Carybdea marsupialis*. Within *Chiropsalmus quadrumanus*, the vector length from the middle to the oral region decreases by an order of six.

**Figure 21.** Vector diagram for *Carybdea marsupialis*. (a) The rhopalial stalk was divided into three regions, the aboral, middle and oral. (b) The protractor provides a reference for orientation. Direction of arrows designates which quadrant, while the length indicates percent area of muscle ( $0.015 \mu\text{m} = 1 \text{ cm}$ ). Directional vectors were subtracted from each other to obtain total vector movement. The net vector orientation is given in terms of final direction (orientation) for each region and the length of the resultant vector (magnitude).

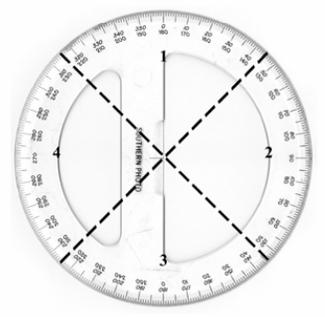
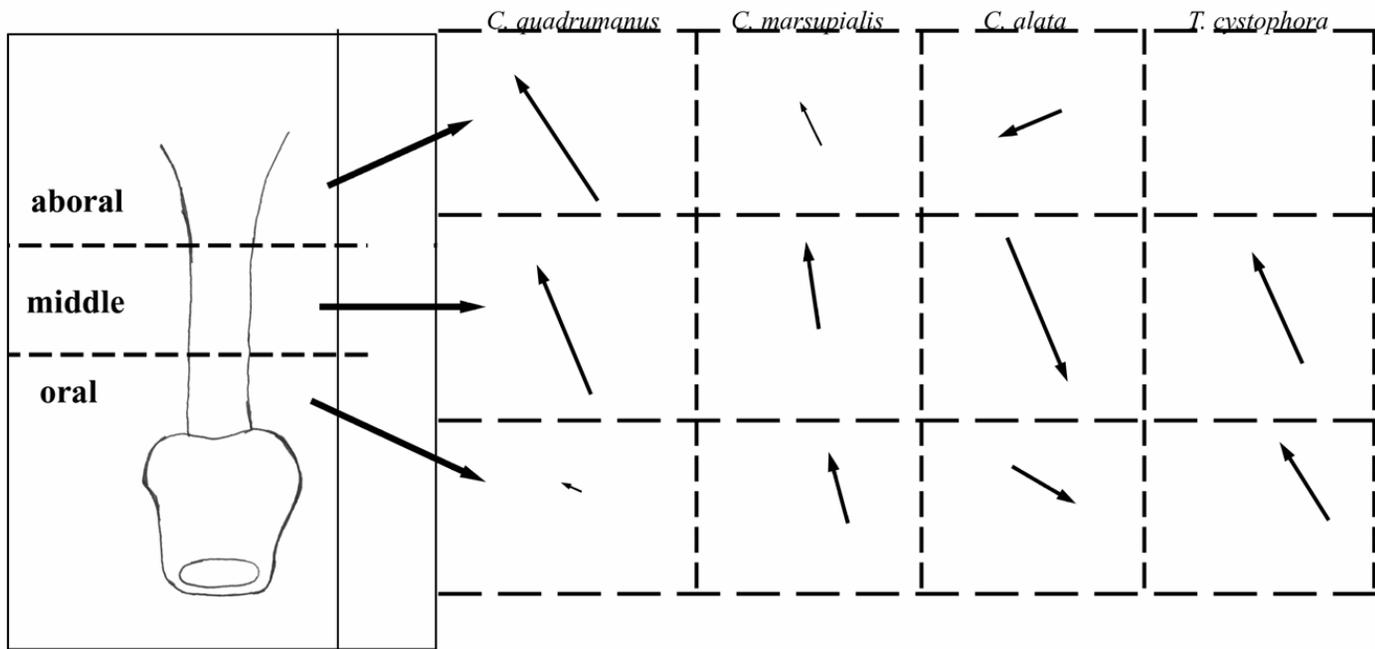
*Carybdea marsupialis*



(0.015  $\mu\text{m}$  = 1cm)



**Figure 22.** Summary diagram for directional vectors for *Chiropsalmus quadrumanus*, *Carybdea marsupialis*, *Carybdea alata*, and *Tripedalia cystophora* in each region. The rhopalial stalk was divided into three regions, the aboral, middle and oral. The protractor provides a reference for orientation. Directional vectors were subtracted from each other to obtain total contractile movement, summarized here only final vector direction, indicated by the arrow. Direction of arrows designates the quadrant, while the length indicates the percent area of muscle fibers ( $0.015\mu\text{m} = 1\text{cm}$ ). Note the difference in scale for the oral region of *Tripedalia cystophora* ( $0.1\mu\text{m} = 1\text{cm}$ ). The aboral region of *Tripedalia* was not included due to the absence of samples.



## DISCUSSION

The rhopalial stalk of *Carybdea alata*, *C. marsupialis*, *Chiropsalmus quadrumanus*, and *Tripedalia cystophora* was investigated by transmission electron microscopy (TEM), immunohistochemistry and light microscopy. The stalk consists of three layers surrounding a central cavity, a branch of the gastrovascular cavity. The epidermis is the outermost layer, followed by the mesoglea and the gastrodermis. The muscular organization of the stalk was examined to determine the role of the stalk and any involvement in a protective response, including the crumple response.

### **Neurite organization**

An epidermal nerve (EN) was present in all four species and consists of two parallel nerve bundles running the length of the stalk, from the aboral to the oral end where the neurites spread out into the rhopalium. The neurites were not followed at length inside the rhopalium. Garm et al. (2006) has previously shown that this nerve is an extension of the ring nerve. Tubulin immunohistochemistry supports the idea that the EN is an extension of the nerve ring, as the labeling clearly indicates the joining of the EN to the nerve ring. The epithelial cells within and around the EN contain a nucleus, few organelles and few muscle fibrils.

A mesogleal nerve (MN) has not been previously noted, although Garm et al. (2006) described a gastrodermal nerve. Whether these two nerves are the same cannot be answered as the nerve observed here in *Carybdea alata*, *C. marsupialis* and *T. cystophora* remains separated from that of the gastrodermis. The nerve is wholly within the mesoglea and although it is variable in its composition, it appears branched in areas of the stalk in *T. cystophora*. When seen in *Carybdea alata* and *C. marsupialis*, it is not as notable as *T. cystophora* and appears smaller in

size and composition. The nerve does not travel the whole length of the stalk, but tapers off just outside the rhopalium. Neurites may be leaving the nerve and contacting both the gastrodermis and the epidermis, possibly the epidermal nerve, but this was not observed due to the small size of the neurites. Garm et al. (2006) observed a gastrodermal nerve, proposing that it was mechanosensory in nature but this nerve was conjoined to the gastrodermis, whereas here it was found to be inside the mesoglea. Additionally, the mesogleal nerve was not found in *Chiropsalmus quadrumanus* and whether this is a family difference or not remains to be seen.

### **Implications of muscle fiber percent area and density**

Longitudinal muscle fibers were found in the aboral-oral axis, and are only present in the epidermis. It has been suggested before that the rhopalial stalk can swing back and forth similar to a pendulum on a clock and can twist (Martin, 2003), and that the stalk functions in guiding the eyes to look through the opening of the rhopalial niche (Hartwick, 1991). This would require a 180° twist from the normal resting position. However, no oblique or helical muscle fibers were found that would allow a twisting motion. Additionally, the absence of circular muscle indicates that the stalk does not lengthen but can only bend or shorten.

Individual muscle fibers in cross-section were differentially distributed in each quadrant. Quadrants were significantly different in regards to muscle fiber percent, both in species and regions. While earlier it was assumed that the muscle fibers in the stalk would be symmetrically activated, it is important to consider the possibility of differential activation, resulting in one quadrant or section contracting preferentially. This would effectively bend the stalk in different directions to move the rhopalium, most likely into a more protective position.

In addition to considering differential activation, it is important to determine to what extent the rhopalial stalk can actually shorten during contraction. Pantin (1952) reported that the

circular muscle fibers of actinians (sea-anemones) can contract up to 20% of their extended length, similar to many other invertebrate muscles. Assuming that the observed length of the muscle fibers in the rhopalial stalk of the box jellyfish (5  $\mu\text{m}$ ) is the extended length of the muscle fiber, the maximum contractility that can occur for each species can be calculated (Table 6). The stalk length ranges from 150-430  $\mu\text{m}$ , with a maximum contraction of 86  $\mu\text{m}$ , assuming that the stalk is only shortening and not bending.

*Tripedalia cystophora*. Although the opening of the rhopalial niche was not observed in *Tripedalia cystophora*, symmetrical contraction of the stalk would move the rhopalium up and away from the opening, pulling it into a more protected position. The maximum shortening of the stalk would be between 30 and 50  $\mu\text{m}$ , based upon a previously reported stalk length (Garm et al., 2006). The oral region of *T. cystophora* had a significantly greater percent area of muscle than either the middle region or any other species. Although this could not be compared statistically, the vector diagrams exemplify the difference in magnitude. *T. cystophora* is found among mangrove roots, increasing the likelihood of potentially injurious stimuli, possibly relating to a higher percent area of muscle fibers. Additionally, the individual fiber size of *T. cystophora*, as shown in the oral region of the histogram (Figure 12), is slightly larger than the other sizes. The size may be increased due to the increased risk of contacting obstacles in the environment. As *T. cystophora* is smaller in size than the other species, the muscle fibers here constitute a larger proportion of their rhopalial stalk. The fibers splay out into the subumbrella, thus when the crumple response is initiated, the rhopalial stalk curls the rhopalium inward, supplementing this protective response. This species is different from the others in that it is the only one found among mangrove roots, while the other three species investigated are found in the open water, off of sandy beaches.

**Table 6.** Stalk length ( $\mu\text{m} \pm \text{SEM}$ ) and maximum contraction ( $\mu\text{m}$ ) of the rhopalial stalk. The maximal shortening is calculated assuming that the observed length of the muscle fibers is the extended length ( $5 \mu\text{m}$ ), and assuming that the muscle fibers are contracting to some 20% of their extended length (Pantin, 1952).

\*The range of the stalk length for *Tripedalia cystophora* given was reported by Garm et al. (2006), thus providing a range for maximum contraction.

Species	Stalk Length ( $\mu\text{m} \pm \text{SEM}$ )	Maximum Contractility ( $\mu\text{m}$ )
<i>Carybdea marsupialis</i>	$430 \pm 21$	86
<i>Chiropsalmus quadrumanus</i>	$400 \pm 48$	80
<i>Carybdea alata</i>	$280 \pm 24$	56
<i>Tripedalia cystophora</i>	150-250*	30-50

*Chiropsalmus quadrumanus*. The symmetrical contraction of the muscle fibers in the stalk would move the rhopalium back into the niche, away from the opening. The hood in *Chiropsalmus quadrumanus* significantly decreases the opening of the rhopalial niche, behind which the rhopalium can be efficiently protected. The magnitude of contraction significantly decreases from the aboral to the oral region, more so from the middle to the oral region. In the other three species investigated, magnitude increases from the aboral to the oral end of the stalk. The total directional contraction for *Chiropsalmus quadrumanus* is towards the back of the niche, but the reason for why the magnitude would be the opposite of the general trend is not clear. The maximum shortening is approximately 80  $\mu\text{m}$ , or 1/5 the length of the stalk. While the stalk would move the rhopalium towards the back of the niche, it may also be shortening to move it behind the hood of the rhopalial niche. Additionally, individual muscle fiber size is generally smaller than that of *Tripedalia cystophora*, but is greater than that of *Carybdea marsupialis* and *C. alata*. Fiber size may not have such a significant effect in *Chiropsalmus quadrumanus*, as the size also decreases from the aboral to the oral region, implying that the oral region may not have a large role in the protective response.

*Carybdea marsupialis*. The rhopalial niche opening in *Carybdea marsupialis* is heart-shaped with a hood curving down in the middle. The lengths of the vectors increase from the aboral to the oral region, although muscle fiber percent area in the oral region is slightly less than that of the middle region. The individual fiber size in *C. marsupialis* is smaller than both *T. cystophora* and *Chiropsalmus quadrumanus*, but generally decreases from the aboral region to the oral region. The loss of individual fiber area may account for the difference in magnitude, but most likely will not have a significant effect. The difference is presumably so small that the contraction will remain the strongest in the oral and middle regions. The summary diagram for

the vectors illustrate that *C. marsupialis* had the smallest muscle fiber percent area. However, *C. marsupialis* had the largest stalk length, a smaller body size than either *Carybdea alata* or *Chiropsalmus quadrumanus*, and a maximum contraction of 86  $\mu\text{m}$ , presumably enough to move the rhopalium behind the observed hood of the cavity.

*Carybdea alata*. Vector length was increased in the middle region, and approximately the same on either the aboral and oral end. However, the percent of muscle fibers in the stalk is more concentrated around quadrant 3 and individual fiber size is significantly smaller than that of *Carybdea marsupialis* and closer in size to that of *Chiropsalmus quadrumanus*. The higher percent area of muscle fibers in quadrant 3 indicates that a contraction would bend the rhopalium towards the opening of the cavity, rather than away or towards the back of the niche. However, the opening of the niche in *Carybdea alata* is small and slit-shaped thus any movement of the rhopalium may not change the protection already afforded. This is also supported by the smaller size of individual muscle fibers, as movement away from the niche may not be as important as it would be in *Tripedalia cystophora*. This supports the idea that the structure of the rhopalial niche may be all the protection needed. Furthermore, the maximum contraction of the rhopalial stalk in *Carybdea alata* is approximately 56  $\mu\text{m}$ . While miniscule in theory, this movement may shorten the stalk enough to move it closer to the roof of the niche, slightly above the opening to the cavity.

## **Conclusions**

The rhopalial stalk of the box jellyfish has been shown to possess the muscular organization to move the rhopalium into a more protective position in three out of four species. In *Carybdea alata*, the stalk moves the rhopalium towards the opening of the cavity, but the rhopalial niche opening may be so narrow as to serve the protective function.

While the rhopalial stalk does possess the muscular organization to move the rhopalium into a more protective position, what remains to be investigated is the role of the statolith in this movement. The statolith is a crystalline-concretion found at the bottom of the rhopalium. It has been proposed both as a gravity-sensing device and to ensure constant orientation of the rhopalium. The stalk may bend in response to the movement of the rhopalium that is weighted down by the statolith. Further investigation into the role of the statolith, together with the movement of the stalk, will lend further insight into the complex behavior of the box jellyfish.

Only longitudinal muscle fibers are present in the rhopalial stalk of the box jellyfish, thus only shortening or bending can occur. No twisting movements would be produced as oblique or transverse muscle is absent, unless passive properties in the stalk allow for it. The absence of circular muscle indicates that no active lengthening can occur, only passive lengthening via relaxation. Furthermore, twisting the rhopalium to “look out” through the niche opening is unlikely as the muscle fibers are smooth. Therefore, relatively slow adjustments occur and medusae are not likely to track moving visual stimuli. It is necessary to consider the role of the smooth muscle fibers found here to be more postural in function, maintaining a constant position in the niche regardless of swimming movements or orientation in the water column. It would be interesting and important to determine to what extent the muscle fibers in the rhopalial stalk can shorten. Pantin (1952) reported that like many invertebrate muscles, the circular muscle fibers of actinians (sea-anemones) can contract approximately 20% of their extended length, with a resting length anywhere between these limits. Therefore, with a smooth muscle fiber length of approximately 5  $\mu\text{m}$ , and assuming that the smooth muscle of the box jellyfish would contract in nearly the same manner, the maximal contraction would be 1/5 the length of the stalk. However, this movement may be all that is needed to move the rhopalium into a more protective position.

Based solely on morphology, the stalk would not be able to twist to look out through the niche or track moving stimuli. However, outside of the stalk nerves, nerve nets extend throughout the stalk epidermis. Directional responses would require differential activation of this nerve net or multiple parallel nets in the different quadrants. Now that the background anatomy is known, behavioral and physiological experiments are needed to answer other questions.

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