



MASTERARBEIT / MASTERS THESIS

Titel der Masterarbeit / Title of the Master's Thesis

COMPARISON of VENOM SEGREGATION by ANTERIOR and POSTERIOR VENOM GLANDS of OCTOPUS MIMUS and CISTOPUS TAIWANICUS

verfasst von / submitted by

Johannes Meirer, BSc

angestrebter akademischer Grad / in partial fulfillment of the requirements for the
degree of

Masters of Science (MSc)

Wien, 2016 / Vienna, 2016

Studienkennzahl lt. Studienblatt /
degree program code as it appears on
the student record sheet.

A 066 831

Studienrichtung lt. Studienblatt /
degree program as it appears on
the student record sheet

Masterstudium Zoologie

Betreut von / Supervisor:

a.o. Univ.- Prof. Dr. Gerhard
Steiner

INDEX

1. Introduction	4
1.1. Understanding of venom and poison and its importance in nature	5
1.2. The World of Cephalopods.....	9
1.2.1. Cephalopod anatomy	10
1.2.2. The Funnel	13
1.2.3. The Visceral Sack.....	13
1.2.4. The Mantle.....	13
1.2.5. The Nervous System.....	14
1.2.6. Importance of the Alimentary Tract of Cephalopods and other	
Molluscs.....	17
1.2.7. Venom of Octopods	21
2. Material and Methods.....	24
2.1. Sample collection and preparation	25
2.2. Protein extraction.....	27
2.3. Protein analyses	27
2.3.1 Acetone precipitation	27
2.3.2. SDS-Page	28
2.3.3. Highly sensitive Liquid Chromatography combined with Tandem Mass	
Spectrometry (LC-MS/MS) and the Shotgun Proteomic-analysis	29
2.4. Morphology	29
3. Results.....	30
3.1. Samples.....	30

3.2. SDS-PAGE	33
3.3. Highly sensitive Liquid Chromatography combined with Tandem Mass Spectrometry (LC-MS/MS) and the Shotgun Proteomic-analysis	34
4. Discussion	46
Results and influencing factors	46
Source of supply.....	47
Techniques	48
5. Summary	49
6. List of figures and tables.....	50
EXPERIMENTAL PART	51
REFERENCES.....	57
ACKNOWLEDGMENT	63

1. Introduction

Fundamental research is always the very first step on the way of understanding processes in nature. Many times knowledge was gained in a disorderly, not necessarily linear but creative and spontaneous way, based on an idea. At this time, the time of awareness, it is not self-evident to place each single aspect of the new knowledge in a particular context although of course the scientists are doing purposeful research. Every new study, every new result builds the foundation for additional studies and questions together with the eagerly anticipated answers.

Toxinology is an interdisciplinary science of toxins and a section of Toxicology. It deals with toxins respectively biogenous compounds synthesized or segregated by venomous animals such as snakes, for example *Oxyuranus scutellatus* (Taipoxin) and poisonous animals, for example *Tetraodontidae* (such as pufferfish, segregating Tetrodotoxin) but also plants (poisonous or toxic plants) and microbial organisms, for example bacteria such as *Clostridium botulinum* (botulinum toxin) or fungus (mycotoxins) (Mebs, 2002). This field of research has today acquired an important role in human medicine and pharmacy and contributes not only to the understanding of the complex world of toxins but to develop new methods of treatment (Lewis, 2003, Singer, 2015) and drugs (Kyriacou, 2010).

This study is intended to make one of these first steps to understand the complexity of octopods toxins, to compare the venom glands of octopods and their venom segregation. It is the very first study of this kind that compares not only the right and left side of each (buccal/anterior and esophageal/posterior) salivary gland of Octopods but also the buccal/anterior with the esophageal/posterior salivary glands respectively their segregations. Previous studies have shown that the venoms of Cephalopods can be as complex as the venoms of very well investigated "classical" venomous organisms like snakes, spiders or scorpions and hold enormous potential not only for understanding fundamental aspects of venom evolution but also as an untapped source of novel toxins for the development of new and unconventional approaches of therapies and drug design (Fry, 2009, 2013).

Although the complex LC-MS/MS analysis has not worked as planned due to technical difficulties (no results available) this study confirms the previous assumptions that there

is a difference between the buccal (anterior) and esophageal (posterior) salivary glands and no difference between each right and left side of the salivary glands.

1.1. Understanding of venom and poison and its importance in nature

It is necessary to explain the meaning of the terms "venom" or "poison" and "toxins" or "toxicants" as they are used in the following.

"Poisons" or "venoms" are all those substances which damage an organism once particular thresholds are reached. In case of venomous or poisonous animals venom or poison is not just one single substance but also a conglomerate of up to 200 different, active components (for example cone snails - conotoxins) (Terlau, 2004).

From this perspective, the poison of bees, toads or snakes are all such *mélanges* consisting of many different components including toxins. How complex a venom can be is vividly illustrated by the venom of the cone snail *Conus geographus* **Fig. 2**. Although the venom of octopuses is not that complex, it should however be emphasized that this example of the venom of *Conus geographus* is intended to provide a better understanding of how complex a venom can be. (Mebs, 2002)

"Toxins" are substances that are generally of natural origin, chemically pure and uniquely defined. "Toxicants" are synthetic substances like toxic chemicals. A dilemma begins if one is trying to separate highly potent toxins from non venomous enzymes.

For example bee venom contains among others the toxins "Melittin" and "Apamin" as well the nonvenomous enzymes "Hyaluronidases" and "Phospholipases A2". Exactly the "Phospholipases A2", in venoms the most common enzyme, is just able to cause in combination with other components a variety of effects like Myo-, Neuro- or Cardiotoxicity which are not necessarily linked with the enzymatic activity. Despite the numerous toxins and even though they are very different in their chemical structures, the most effective ones are always targeting the most important vital function: the nervous system. (Mebs, 2002)

Venom systems are widely used in the animal and plant kingdom. They are key evolutionary innovations in a broad phylogenetic range of animal lineages and a large range of opportunities is available to make use of them, either for predation, defense, or deterrence. Snakes, scorpions, and spiders represent the most extensively studied and medically important clades but the "world of venomous and poisonous animals" also

includes several insect orders, lizards, fish, echinoderms, centipedes, cephalopods, jellyfish, anemones, sea snails, and even some mammals (platypus and shrews). It does not matter whether by poison fangs, poison stings, barbs, sprays, beaks, harpoons, pinchers, spurs or nematocysts, venom delivery systems sometimes show great variation in complexity and structure and the targets are always the same: all major physiological pathways and tissue types are accessible by the bloodstream. After a direct encounter with another organism either intentionally or unintentionally an envenomation may occur. Moreover, even if poisonous marine animals or such which unexpectedly got poisonous (for example adulterated seafood) are consumed, severe envenomations respectively poisonings can be caused. Suchlike envenomations can be of trivial nature and just impair our well-being, for example through pain but it is not uncommon that severe consequences occur with a sometimes fatal outcome.

In everyday language animals that produce poison are designated as "poisonous animals" but things are not quite as simple as that. On one hand there is a large variety of animals which not only produce poison but also use it actively (hunt or defense) and are able to administer poison very targeted like for example snakes, spiders, or scorpions. On the other hand a large variety of animals exists that as well produce poison but without such useful tools like teeth or stings. The latter, for example toads like *Bufo marinus*, are able to produce poison in glands and accumulate it in specific organs like their skin. Other animals are not able to produce poison themselves but modify substances found in their prey. Poison frogs (*Dendrobatidae*), which feed on specific arthropods consume the needed components through these and segregate toxic alkaloids through their skin. This way most lineages of poison frogs are not producing alkaloids by themselves but appear to accumulate them from a natural diet of alkaloid-containing arthropods which include certain mites, ants, millipedes and beetles (Saporito, 2007).

It is quite complex to characterize the term "poisonous animal" but nevertheless two groups can be set up: "venomous animals" and "poisonous animals". In simple terms, the former are organisms that are able to produce and administer poison with the help of a tool, the latter are not. Under these circumstances it is no longer called poison but venom (venomous animals). This "venom apparatus" is composed of venom-producing and -storing gland tissue which is connected with a "transmission apparatus" like a

sting, specialized fangs or jaws with grooves through which the venom is administered into the bite wound (**Fig. 1**). An important aspect in this context is that the secretion of the venom gland (the venom) is used parenterally, this means it enters the blood circulation of the prey or victim without prior crossing the alimentary tract (risk of an inactivation) (Mebs, 2002, Fry, 2009).

However, there is no clear separation between "venomous" and "poisonous animals" because for example a lot of toads or salamanders have venom glands and some of them are able to spread their poison actively but miss fangs or other "venom tools". This example shows particularly well how difficult it is to make a strict classification of "venomous animals" and "poisonous animals" if a specific structure, like a sting or fang to administer venom parenterally, is missing. Such poisons then have to be absorbed via the alimentary tract, respectively enteral, which would make it entirely possible to classify amphibians as "venomous animals" (Mebs, 2002).

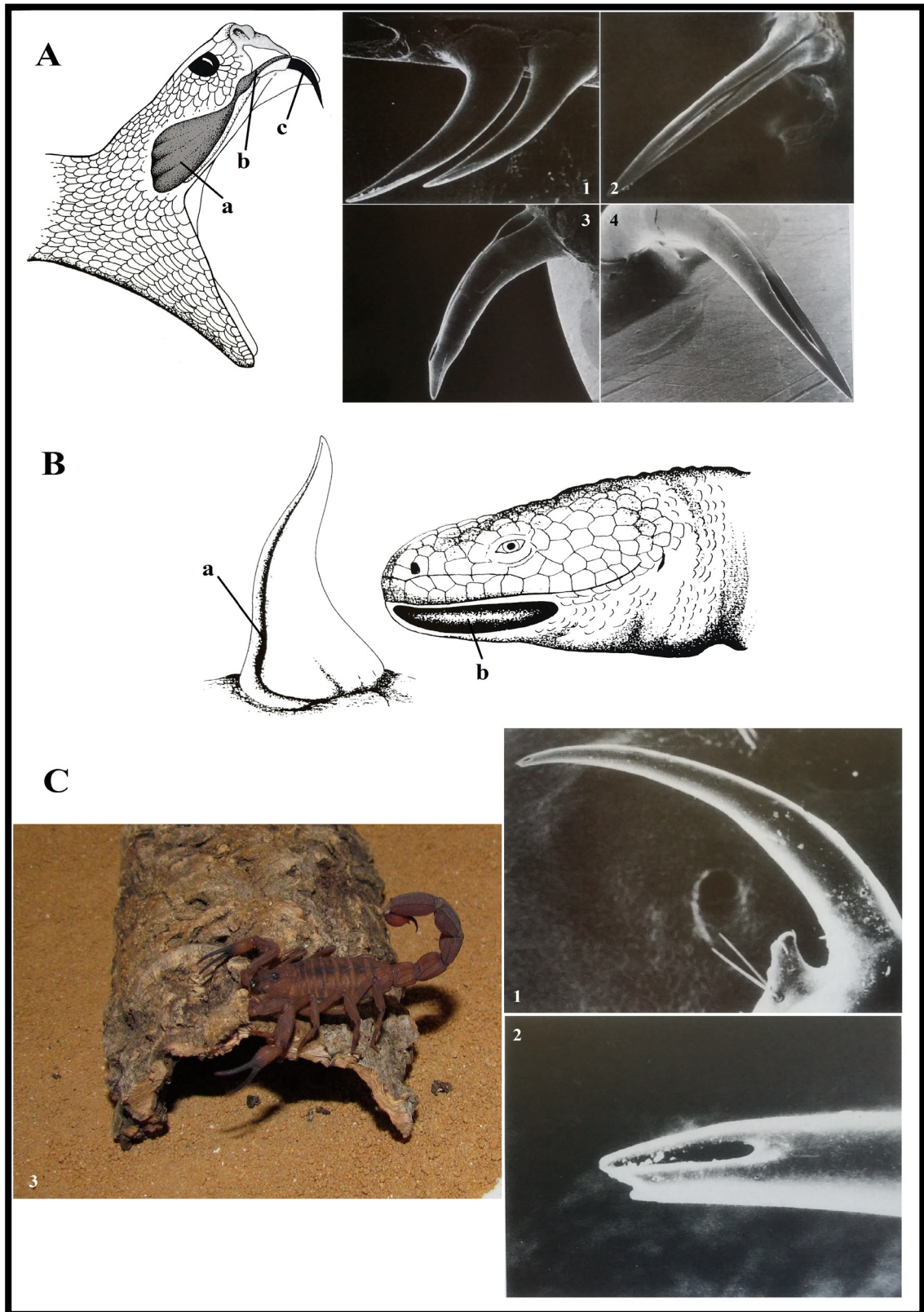


Fig 1: (A) Schema of the location of the venom gland in a pit viper, a, venom gland, b, venom duct, c, fang (Mebs, 2002); 1 - 4, Scanning electron micrographs of different kinds of fangs, (1) (50x) Aglyphous teeth (lacking grooves) of *Natrix natrix*, (2) (30x) Opisthoglyphous teeth (rearward grooves) of *Thelotornis kirtlandii*, (3) (24x) Proteroglyphous teeth (forward grooved) of *Naja nigricollis*, (4) (26x) Solenoglyphous teeth (pipe grooved) of *Bothrops atrox* (picture 1-4 Mebs, 2002) (B) Scheme of the head and teeth of a venomous lizard, *Heloderma suspectum* (Gila monster), b, the venom glands are located in the lower jaws; a, the teeth have two grooves through which saliva mixed with venom can flow into the bite wound (Mebs, 2002). (C) Scanning electron micrographs of the sting of a Scorpion (*Babycurus jacksoni*), (1) (30x) Sting and (2) (300x) excretory duct (picture 1, 2 Mebs, 2002)

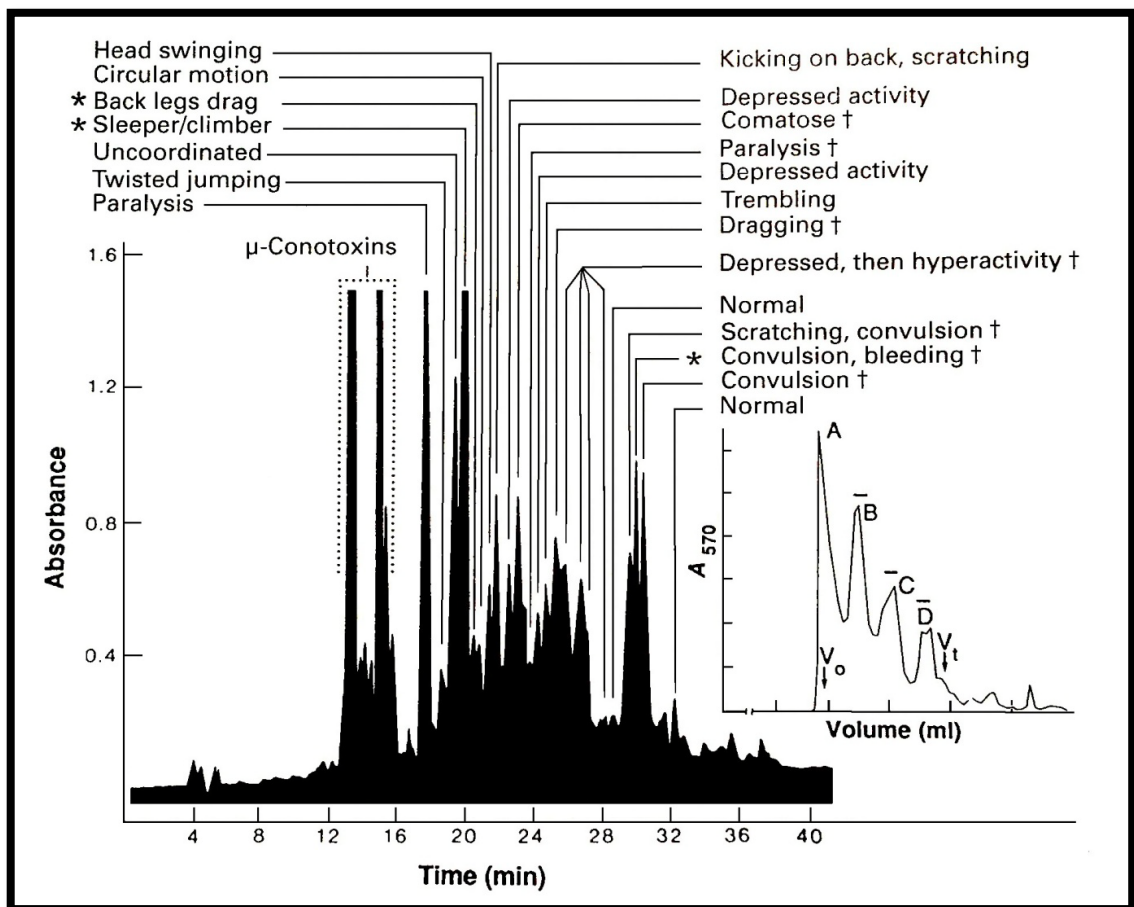


Fig. 2: The venom of a cone snail, *Conus geographus*, includes a variety of toxins with different modes of action. First the venom was separated in 4 main groups using gel-filtration (right) from which of group B, using high-pressure liquid chromatography (HPLC), numerous toxins were isolated which produce different effects in mice; V, volume; V₀, void volume (volume of liquid after the first main groups appear); V_t, rest (mostly dirt); A₅₇₀, wavelength (570nm) - note: because ninhydrin was used for the protein screening the used wavelength was 570nm but that today 220nm are the norm. (Mebs, 2002)

1.2. The World of Cephalopods

The name "*Cephalopoda*" was introduced by Georges Cuvier 1797 and replaced the much older nomenclature "*polyp*" (πολύπους polýpous „*polypod*“) used by the ancient authors like Aristotle and Pliny the Elder.

The *Cephalopoda* are representing an ancient and very successful group of the phylum *Mollusca*. Over various times in geological history these exclusively marine animals have been among the dominant large predators in the ocean. They are highly developed *Conchifera* (subphylum of the phylum *Mollusca*) whose sensory functions may outperform those of the remaining invertebrates. Containing the *Nautiloidea* with a few species of the pearly nautilus and the *Coleoidea* including the squids, cuttlefish,

octopods and vampire squids, two groups of cephalopods exist today (Westheide, 2007).

"With approximately 800 known species, coleoids represent an important element in marine trophic systems worldwide, displaying an impressive variation in shape, size (from 2 cm to over 10 m) and ecology (benthic to abyssal, tropical to Antarctic)." (Fry, 2015).

These exclusively marine animals occur nowadays in all oceans of the world but became dominant during the Ordovician period (~485.4 - 443.4 mio. y. ago). The *Cephalopoda* are the most active of all the molluscs and especially the squids are able to compete against other highly developed predators like fishes in their swimming speed and ability of catching prey. They occupy a wide variety of habitats in all of the world's oceans and were found in great depths of up to approx. 5500 m (*Chroteuthis veranyi*) but will probably reach much greater depths (Westheide, 2007).

Although the genealogical tree is still not fully clarified one can assume that they first appeared around 500 million years ago in the "Upper Cambrian Period" and two extant lineages may have separated around 470 million years ago. The long separation of the two lineages has, today, resulted in lineages with cephalopods that are very different in structure (Westheide, 2007).

Due to this long evolutionary process they developed not only a highly developed repertoire of sensory functions but are also able to achieve excellent physical performances. Along with benthic species there are numerous pelagic species that generally are excellent swimmers. Some pelagic species appear to be cosmopolitans; not a few display periodic daily vertical migrations in search of food (*Dosidicus gigas*, *Nautiloidea*) - due to seasonal influences - or cover long spawning migrations (Westheide, 2007).

Nevertheless looking back over the last decades, research on Cephalopods was focused on the nervous system and their sensory functions and very little on their venoms.

1.2.1. Cephalopod anatomy

Cephalopoda are largely bilateral symmetric emphasizing the dorsoventral axis that is tilted by 90° in the horizontal so that the morphological front side becomes the functional top side. The *cephalopodium* (noun; Greek *kephale*, head; *pous*, foot) (head-

arm-funnel-complex) forms a functional unit for locomotion and catching prey and is distinctly separated from the remaining body (Maggenti, 2011). Comparable with arthropods and vertebrates there is strong tendency for cephalisation and cerebralisation (Westheide, 2007).

One or two rows of tentacles are arranged around the oral aperture and "*A funnel derived from the molluscan foot or the molluscan neck region.*" (Salvini-Plawen, 1980, Shigeno, 2008).

The arms are strongly differentiated and serve different functions; the movement is done by a "muscle-antagonist-system" similar to the tongue of the *Craniota*. Among the *Nautiloidea* they number naturally 90 tentacles placed around the oral aperture, each of them is retractable into its sheathed base (**fig. 3 and 4**). *Coleoidea* have 8 or 10 arms. On the side facing the mouth each arm has one, two or four series of suction cups, each of which mounted with a broad basis in the case of the *Octopoda*. In case of the *Decapodiformes* the suction cups have a pedunculated basis enclosed with toothed rings of chitin which turned into grab hooks for certain species. Onshore both, the *Octopoda* and *Decapodiformes* are able to move on respectively by means of their arms. The latter have ten arms, two of them act as exceedingly long feeding tentacles. One modified arm of the male, the *hectocotylus*, is transferring the spermatophores to the female (Westheide, 2007).

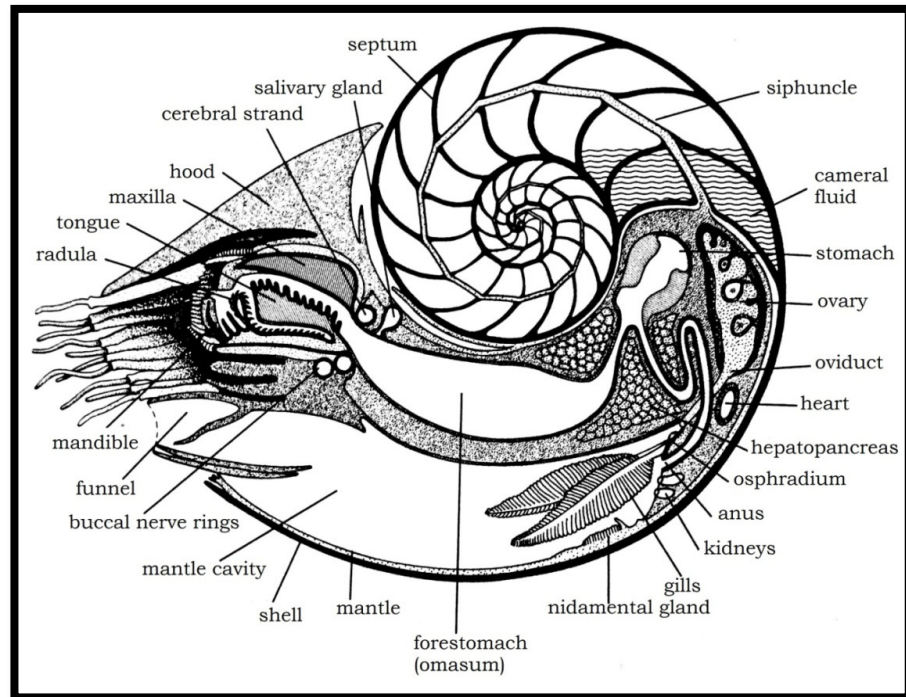


Fig.3: *Nautiloidea* - scheme of the inner organisation. The mantle is helical curved and forming an outer casing. The animal occupies the front/broader parts of the shell (living chamber), the parts which are no longer inhabited are locked by septa. The siphuncle traverses the rearmost part of the shell, controls the buoyancy and equalizes the hydrostatic pressure by changing the amount of fluid or gas. This way the shell becomes a hydrostatic organ. (Wilbur, 1988)

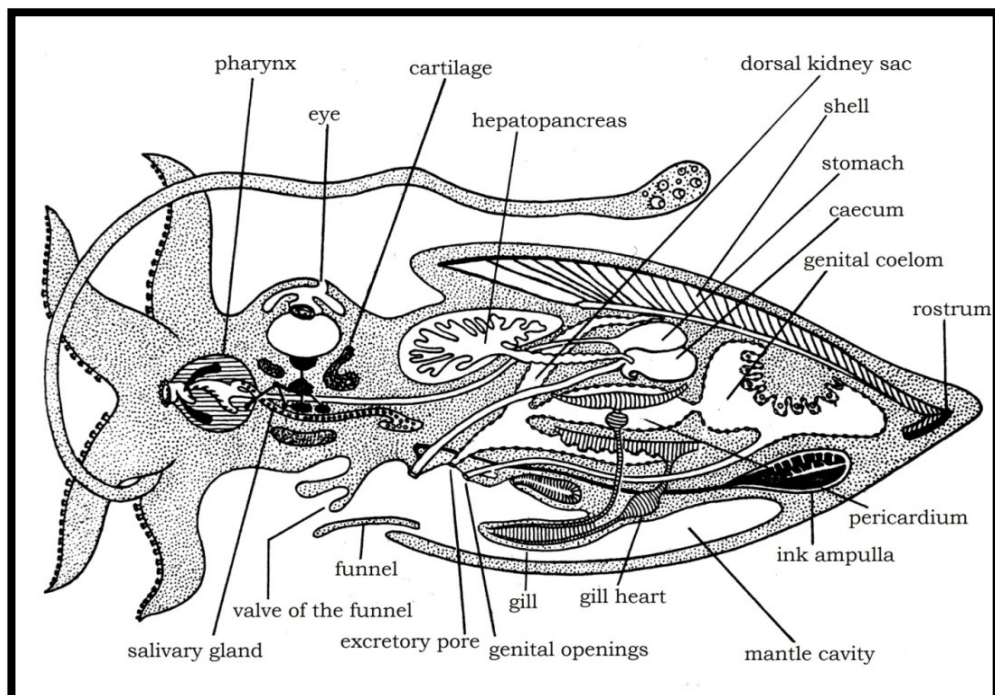


Fig.4: *Coleoidea* (*Sepia officinalis*). Scheme of the inner organisation - longitudinal section. The left eye did turn upward by approx. 90°; most important bloodstreams are shaded, coelom and kidneys white with schematized epithelium. By Stempell (1929) off Kaestner (1969). (Wilbur, 1988)

1.2.2. The Funnel

Behind the crown of arms as a foot derivative the funnel forms a tube-shaped, movable connection between the spacious mantle cavity and the environment. Based on the jet propulsion principle the body moves by powerful muscle contractions of the mantle, squeezing the water out of the mantle cavity (**fig. 4**). This is being achieved by the musculature of the mantle (**fig. 6**) and the two muscles *Depressor infundibuli* (Storch, 2002). Moving through the water during the escape, the funnel is directed to the head and the physiological hind body (visceral sack) in the front but the funnel can be bent and enables together with the arms versatile manoeuvres. The opening of the mantle cavity is often sealed by a push-button-like locking mechanism, for certain species the rims of this gap are coadunate (**fig. 3, 4**) (Westheide, 2007).

1.2.3. The Visceral Sack

The visceral sack is very large in relation to the remaining body. At *Nautiloidea* and *Spirula (spirula)* the hind body is extended into a thin, tube-like appendix, the siphuncle which leads like a duct through the phragmocone, the chambered portion of the shell of a cephalopod, and every single of its chambers (camera, pl. camerae) (**fig. 3 and 4**). Of the recent *Cephalopoda* just the species of the *Nautiloidea* have an outer shell; regarding the *Coleoidea* the shell is reduced and displaced into the body or has completely disappeared (Westheide, 2007).

1.2.4. The Mantle

In the case of *Nautiloidea* the mantle is a thin epithelium covering the visceral sack whereas in the *Coleoidea* the mantle performs numerous tasks (**fig. 5, 6**). Particularly among the excellent swimmers of the open sea the muscular mantle is a very complex and versatile organ. The integument is formed by a single layer epithelium composed of pillar-, gland- and sensory cells. A basal matrix separates the epithelium from the underlying musculature and connective tissue. Another kind of musculature is also completely covering the arms of the *Coleoidea*, which are stable and freely movable in all directions because of the overlay of the muscle layers with different alignment of the fibers (Westheide, 2007) (**fig. 5, 6**).

1.2.5. The Nervous System

The *Coleoidea* have the highest developed nervous system of all molluscs, even of all invertebrates, and because they cannot withdraw into a protective shell in times of danger and in general are catching nimble prey, they have to react very quickly. Their main ganglia are merged to one complex brain (the highest form of this development can be found at the octopi) which is protected by a cartilaginous capsule. Only in *Nautiloidea* the nerve centers of the head still have the nature of medullary cords. The brain of the *Coleoidea* is constructed of lobes which, according to their function, differ in relative size and internal structure. Especially the optical lobes are much more developed. A very dense network of nerves innervate especially the arms. Ensuing from the cerebral ganglion they pass through the pedal ganglion and form brachial ganglions and arm nerves for each sucker cup. Each sucker cup possesses its own ganglion. In the peripheral area the stellate ganglion (*Ganglion stellatum*) has a particular importance as a "control centre" for the mantle musculature. The Decapodiformes developed a system of giant nerve fibers for a very fast saltatory conduction which enables also a simultaneous contraction of the mantle musculature (Westheide, 2007).

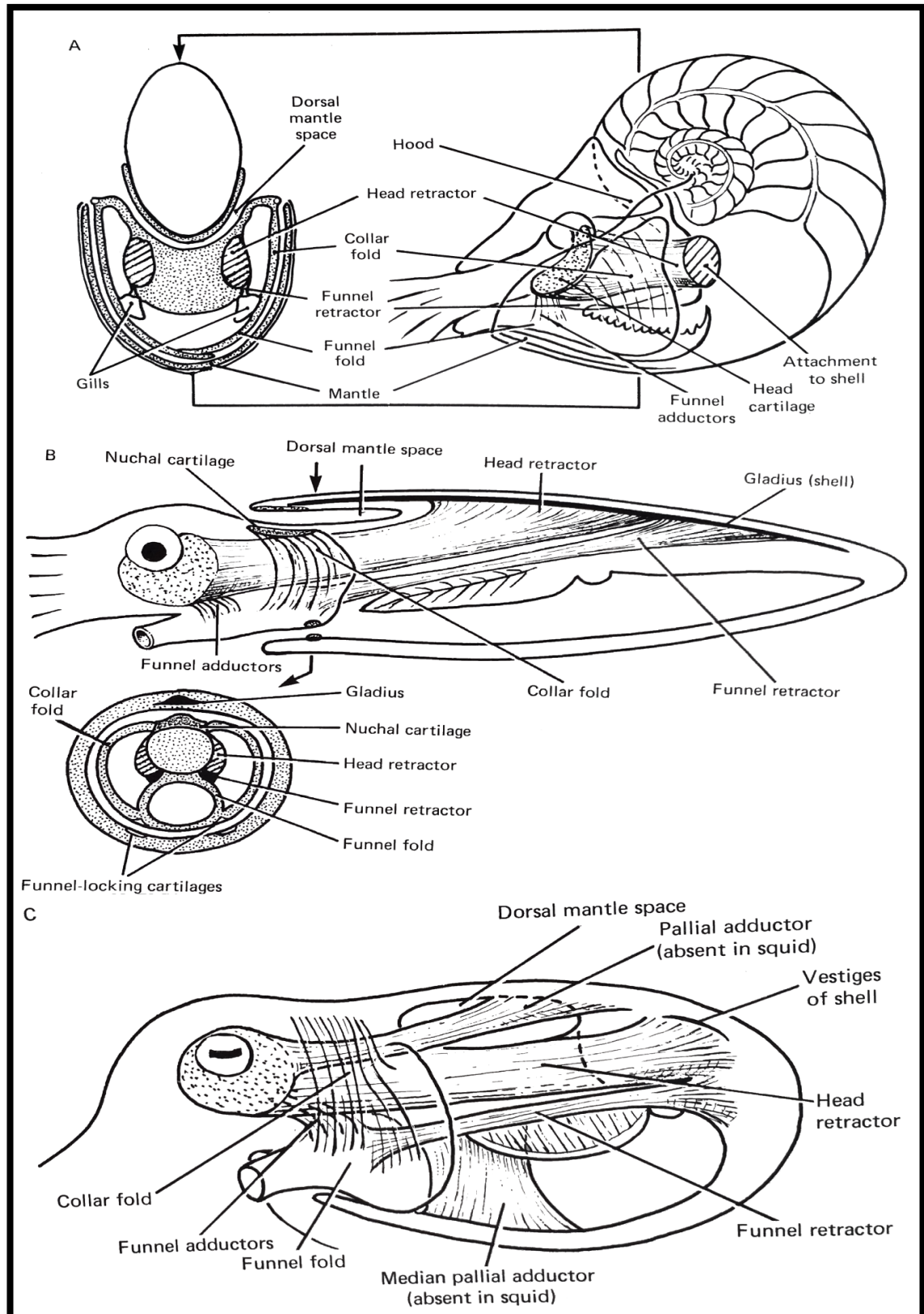


Fig. 5: Muscles associated with the mantle cavity in (A) *Nautilus*; (B) a squid, such as *Loligo*; and (C) *Octopus*. The shell is progressively reduced, becoming internal (and absent in some octopods). Homologous muscles remain, the same elements from the funnel and its wings, and the gills retain a constant relation to other structures, dividing the mantle into paired lateral and single ventral spaces. In octopods the dorsal mantle space has become cut off from in front, but remains in connection with the lateral spaces. There are additional pallial adductor muscles running from the head cartilage to the sides of the mantle and a pair of dorsoventral adductors in the midline, linking the mantle floor to the viscera. (Wilbur, 1988)

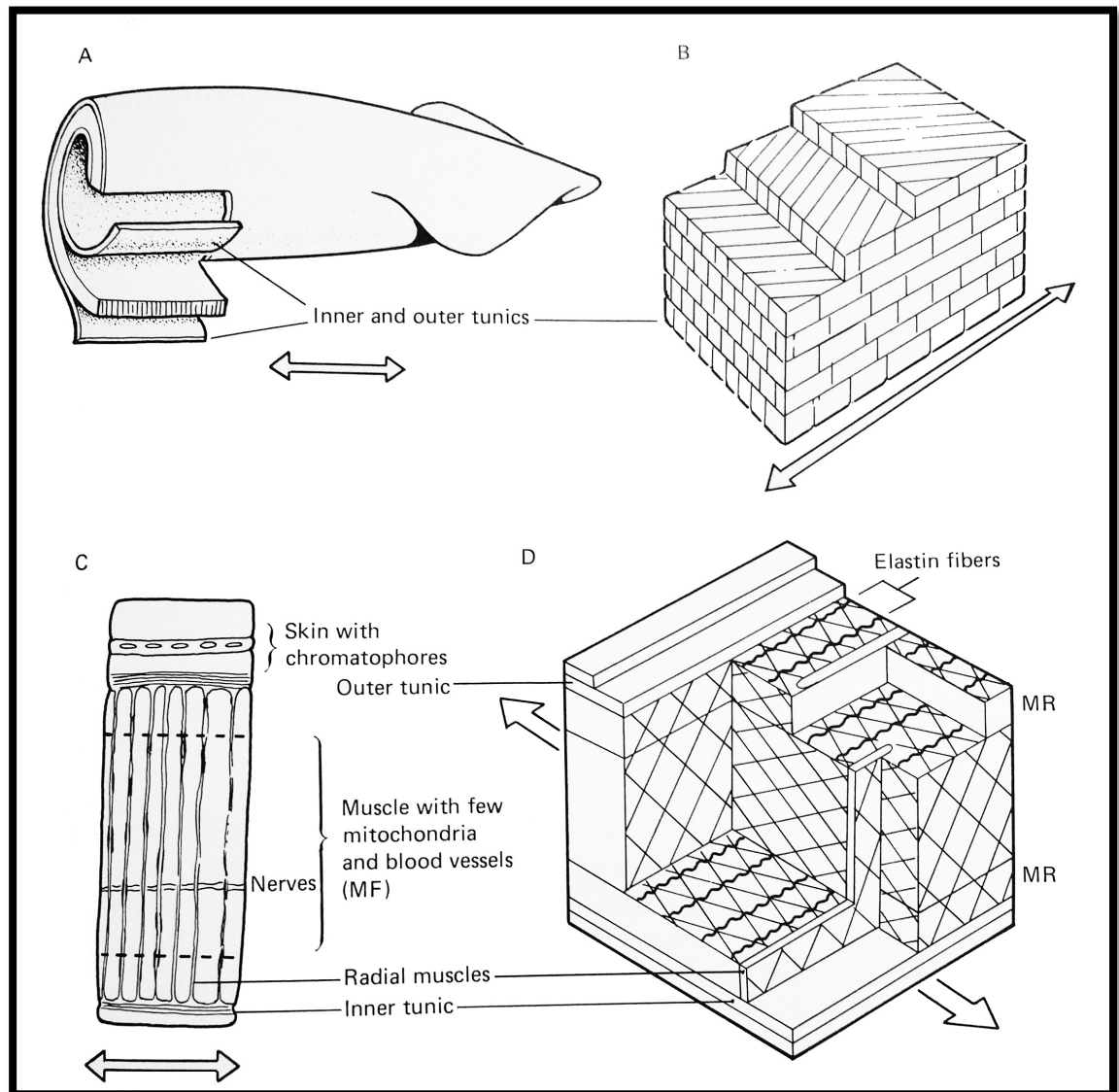


Fig. 6: Mantle structure in squids. (A) and (C) show how the muscle is sandwiched between two collagenous tunics, having the structure shown in (B). (D) gives more details of the intramuscular connective tissue lattices. It also shows how the radial muscles are arranged in sheets parallel to the circulars, and the distribution of the two types of circular muscle: mitochondria plentiful (MR) and mitochondria sparse (MF). In each diagram the open arrows show the longitudinal axis of the animal. [A-C, after Ward and Wainwright (1972; *Loliguncula*); D, after Bone et al. (1981; information from *Alloteuthis*, *Loligo*, and *Sepia* - which also has some longitudinal muscles just inside the connective tissue and epithelial layers lining the mantle).] (Wilbur, 1988)

1.2.6. Importance of the Alimentary Tract of Cephalopods and other Molluscs

The following chapter examines the alimentary tract and its importance for *Siphonopoda* (cephalopods) and other mollusks. Precisely because the alimentary tract is adapted to nutrition and food it affects the segregation of venom (Fry, 2009, 2010).

Over the course of evolution the highly dome-shaped visceral hump of the *Siphonopoda* led to a change of the body axis and resulted in a generally recurving alimentary canal with the stomach located at the most posterior point of the gut. In general, in mollusks development the yolk is absorbed by the primordium of the midgut except in *Siphonopoda* where the embryos differentiate a proper yolk-absorbing circulatory system (Wilbur, 1988).

Within two circles in *Nautiloidea* around 90 slender arms are arranged around the mouth opening and 10 or 8 arms in *Coleoidea*. A special peribuccal membrane, sometimes extended to tips with minute suckers, is present in the decabrachiate groups. A circular smooth lip and a subsequent papillated inner lip are ending directly into the buccal cavity, the anterior-most region of which is occupied by strong jaws. The mid-anterior portions form a beak with these organs distinctly pointed and the lower jaw passes over the upper jaw; "only the latter appears to be homologous to the jaw of other *Conchifera*." (Wilbur, 1988). Starting at the hardened biting hook both jaws enlarge posteriorly into two kinds of lamellas, one short outer lamella and one more extended inner lamella. The jaw musculature fills the space between and is inserted in these lamellas. It is noteworthy that the size of the jaws depends on the body size as well as on the sex and that the shape of the jaws is species specific (Wilbur, 1988).

To cause wounds or drill holes a well adapted radular apparatus is needed. This means that in *Nautiloidea* the radula bears 13 teeth or plates, arranged directly one behind the other in rows respectively nine, seven or five (five in *Gonatidae*) elements in *Coleoidea* except in *Spirula* (*Decabrachia*) and the *Cirroteuthidae* (*Octobrachia*) where the radula is reduced (**Fig 7**) (Solem, 1975).

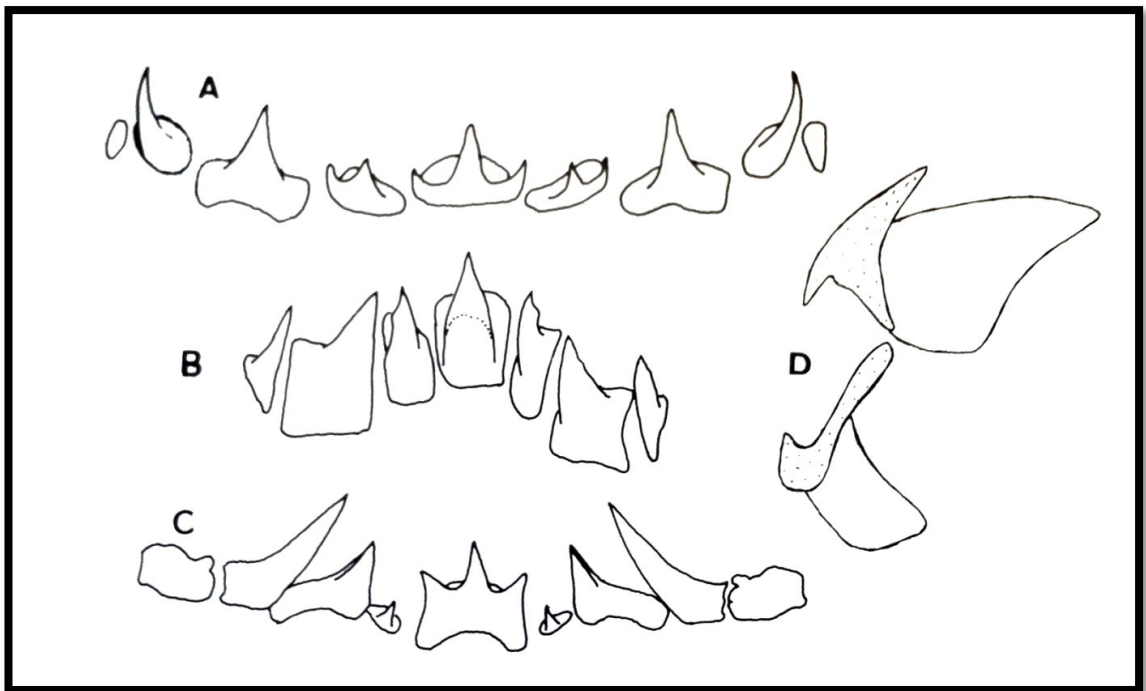


Fig. 7: Radula (A-C, one transverse row each) and jaws (D) in Coleoidea. (A, D) *Loligo pealii* [Teuthida; after Williams (1909)]; (B) *Vampyroteuthis infernalis* [Vampyromorpha; after Pickford (1949)]; (C) *Tremoctopus violaceus* [Octobranchia; after Thomas (1977)]. (Wilbur, 1988)

Furthermore Bidder, A.M. 1966, Fuchs E. 1973 and Salvini-Plawen 1981 already studied intensively these parts of the alimentary tract: "*Four different sets of foregut glandular organs can be recognized. The only glandular organs present in Nautiloidea in the ventrolateral buccal lobes may be homologous to the paired anterior foregut glands at the back of the buccal mass in Coleoidea (lacking in cirromorph Octobranchia), which open above the radula onto the inner faces of the so-called lateral lobes. These are a lateroanterior extension of the posterior buccal epithelium and in most Coleoidea they also bear a chitinous layer with denticles directed toward the pharyngeal lumen between.*" (Wilbur, 1988). Located at the anterior end of the midgut gland the posterior foregut glands (poison glands) open by means of a long common duct on a large papilla below the subradular pouch (they are missing in *Nautilus*, in *Spirula* (*Sepiida*) and in cirromorph *Octobranchia*) (Bidder, 1966) (**Fig. 9**).

In *Octobranchia* and *Nautiloidea* the crop or prestomach is shown as an initial enlargement by the posterior, entodermal esophagus (**Fig. 8**) (Wilbur, 1988).

Groups like *Octopodidae*, whose prey is known to be cunning or potentially dangerous, have to use their venom effectively and targeted. They are known to be opportunistic

and versatile predators and prey on a variety of taxa, including crustaceans, other molluscs, fishes and even sea birds (Grubert, 1999).

An interesting aspect of octopods in catching shelled prey is that they first attempt to pull open crustaceans because this kind of handling is often much faster but also 1.29 times more costly than drilling (Steer, 2003). In case of large or large shelled prey (crustacean) where physical power may not be sufficient the octopus injects its toxic saliva through either puncture wounds or holes caused by the beak or drilled mainly by the physical actions of the radula (Runham, 1997). The toxic saliva is then killing or paralyzing the prey as well as aiding in the detachment of tissue from the exoskeleton (Grisley, 1987, Nixon, 1984, Pilson, 1961, Cortez, 1997).

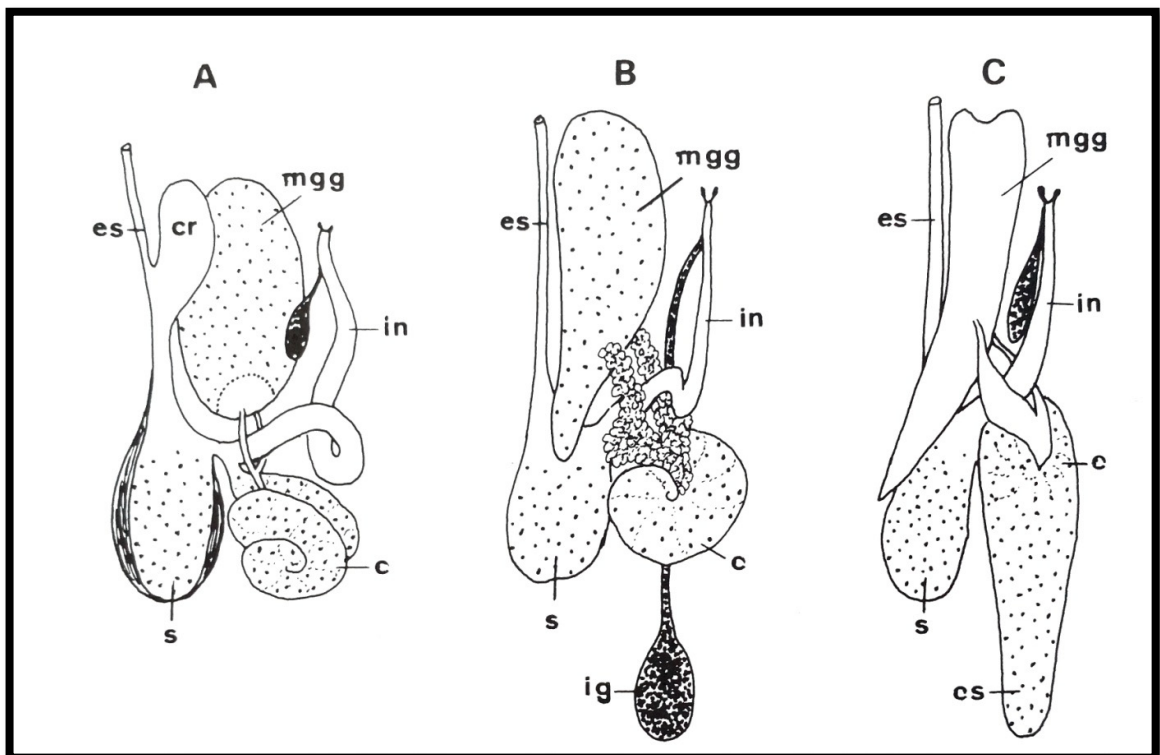


Fig. 8: Alimentary tract in Coleoida [after Bidden (1966)]; stippled, sites of digestion (no absorption in stomach sac, s). (A) Octopus (Octobranchia); (B) Sepia (Sepiida); (C) Loligo (Teuthida). c, Stomach cecum; cr, crop; cs, cecal sac; es, esophagus; ig, ink gland; in, intestine; mgg, midgut gland; s, stomach sac. (Wilbur, 1988)

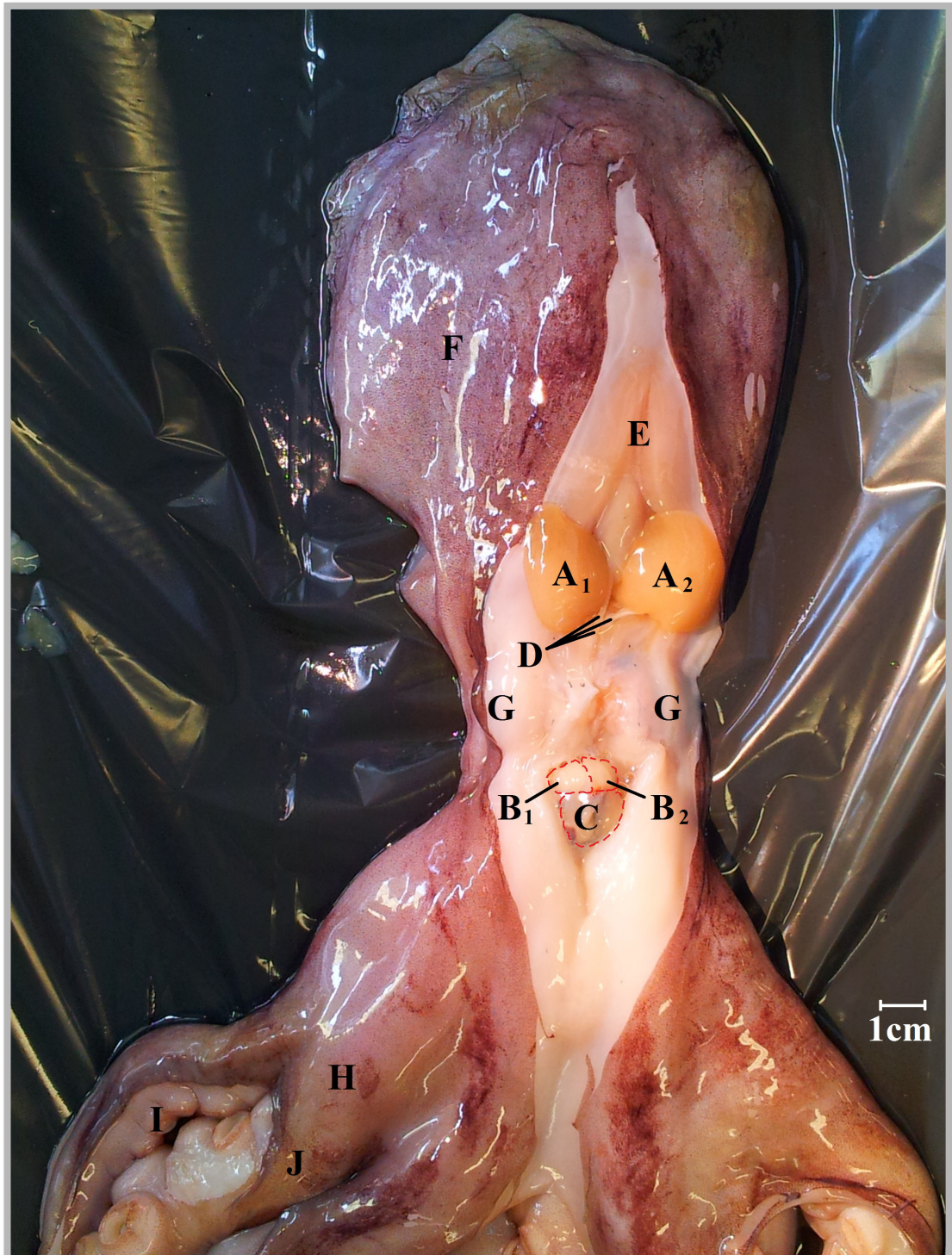


Fig. 9: Dissection of *Octopus mimus* - frontal view. A1, Right Oesophageal Gland (Right PSG - Posterior Salivary Gland); A2, Left Oesophageal Gland (Left PSG - Posterior Salivary Gland); B1, Right Buccal Gland (Right buccal gland - Anterior Salivary Gland (ASG)), B2, Left Buccal Gland (Left buccal gland - Anterior Salivary Gland (ASG)); C, Buccal Mass; D, Common Duct; E, Dorsal Incision; F, Mantle (front side view); G, (right and left) Eye; H, Arm bases; I, Suction Cup; J, Arm.

Versatility and adaptability of cephalopods feeding behavior have played a large role in their evolutionary success (Fiorito, 1999).

1.2.7. Venom of Octopods

Before attacking Octopods have the ability to assess their prey by an excellent developed visual sense whether physical force is sufficient or the usage of venom is needed to overpower the prey and preventing injuries. Cephalopod venom is a conspicuously neglected area with the majority of venom research carried out on snakes, spiders, scorpions and cone snails. (Fry, 2010)

Latest research has shown the venoms of Coleoids (cuttlefish, octopus and squid) are as complex as the venoms of some of the above-mentioned organisms. Furthermore, coleoid venoms have significant potential not only for understanding fundamental aspects of venom evolution but also as an untapped source of novel toxins for use in drug design and discovery (Fry, 2009).

Studies of coleoid venom molecular evolution have shown that octopuses and cuttlefish share a common venomous ancestor (Fry, 2003, 2005, 2009). However, studies have not investigated the differences in venom composition between the anterior and posterior paired glands as well as between their left and right side or the clear allusions to their origins of same species (Fry, 2013). This is precisely what this study focuses on.

Already in 1888 the toxicity of octopus saliva from the posterior pair of salivary glands (PSG/esophageal glands) to invertebrates was established (Lo Bianco, 1888) and later Ghiretti succeeded in isolating the “crab toxic fraction” from *Sepia officinalis*, *Octopus vulgaris* and *Octopus macropus*, which Ghiretti termed Cephalotoxin (Ctx) (Ghiretti, 1960). Although early findings have indicated that the toxin particularly potent against crustaceans (Songdahl, 1974) was a protein, it was assumed for a long time that death or paralysis observed in envenomated prey are caused by the actions of various amines isolated from the octopods esophageal glands (PSG) such as acetylcholine, histamine, octopamine, serotonin and tyramine (Erspamer, 1953, Ghiretti 1960). Studies showed furthermore that in envenomated crabs these amines at realistic concentrations only caused the initial symptoms of overexcitability and additionally were unable to reproduce the irreversible paralyzation achieved by injection of crude saliva. Due to that it was logical to assume a much more complex venom composition (Ghiretti, 1960).

Based on these results Ghiretti noted that Cephalotoxin (Ctx) is a protein-mélange consisting of four proteins including at least one glycoprotein, originally extracted from the esophageal glands (PSG) of the cuttlefish *Sepia officinalis*, and later *Octopus vulgaris* (Ghiretti, 1959, 1960). As mentioned in the beginning such mixtures of toxins have several functions, so has Cephalotoxin a number of activities, including inhibition of respiration in crabs, inhibition of blood coagulation in both crabs and humans and paralyzation of crabs and cockroaches (Ghiretti, 1960, Fry, 2010).

In the course of time octopodid species like *Octopus dofleini* (Songdahl, 1974), *Hapalochlaena maculosa* (Sheumack, 1978), *Eledone cirrhosa* (Grisley, 1993), and *Octopus vulgaris* (Kanda, 2003) gained more and more significance in science, several proteinaceous toxins segregated by the salivary glands have been isolated and their activities described. On one hand encounters between humans and for example members of *Hapalochlaena* with harmful consequences or even human fatalities are well known (Cavazzoni, 2008) but on the other hand these octopods proved very useful in areas like clinical research (Lewis, 2003). Cephalopod toxicological research focused in particular on tetrodotoxin (TTX) and TTX-like compounds (Fry, 2009). However, it has been established that *Hapalochlaena*-TTX is not only distributed in the salivary glands but also in all other parts of the body because it is not segregated by the animal itself but like in many other marine organisms by endosymbiotic bacteria (Yotsu-Yamashita, 2007).

Nevertheless many previous studies of the salivary glands, lacking a closer view at the buccal glands (ASG), have however just revealed that secretions from the esophageal glands (PSG) are toxic and secretions from the buccal glands (ASG) are principally mucous in nature (Gennaro, 1965). However it must be noted that it is entirely ambiguous what kind of relationship exists between these two kinds of salivary glands (Fry, 2009).

In one of the recent studies (Fry, 2009) they investigated inter alia *Hapalochlaena maculosa* and *Octopus kaurana* and found a lot of peptidase S1 transcripts as well as evidence for a basal radiation of these transcripts in *Cephalopoda*. Science may not know exactly about the presumably endogenous salivary toxins found in octopods but it gives reason to suppose that neurotoxins and various proteases are very important venom constituents. Furthermore, multigene toxin families such as conotoxins (Duda,

1999, Fry, 2009) that have undergone adaptive radiation through positive selection cohere to the molecular diversity in the functional residues of the encoded proteins. This functional diversification and basal radiation are consistent with observations of the protease activities in octopodid species belonging to phylogenetically relatively distant subfamilies (Strugnell, 2005) such as the eledonine *Eledone cirrhosa* (Grisley, 1993) and octopodine *Octopus vulgaris* (Morishita, 1974) where scientists could identify ten (at *E. cirrhosa*) and eight (at *O. vulgaris*) caseinolytic proteases (Fry, 2010).

Summarizing, it seems that, like proteases, most of the octopodid venoms share neuro/myotoxins as a common feature. In various studies small neuropeptides and a peptide-transcript (in *Octopus kaurna*) homologous to that of OctTK (Fry, 2009) were found in esophageal glands (PSG) extracts of species belonging to both *Octopodinae* and *Eledoninae*. These neuropeptides like Octopus tachykinins (OctTK) from *Octopus vulgaris* (Kanda, 2003), eledoisin from *Eledone aldrovandi* and *Eledone moschata* (Erspamer, 1962) have hypotensive effects. Simultaneously in the extracts of *Octopus dofleini* (Songdahl, 1974), *Octopus vulgaris* (Ctx) and *Eledone cirrhosa* (McDonald, 1972), non-TTX like neuro/myotoxic proteins have been isolated (Fry, 2010).

In the light of these points and given the fact that recent studies focused only on the esophageal glands (PSG), toxins segregated by this pair of glands are known (**Table 1**) as opposed to the buccal glands (ASG) where just a few transcriptomes are reported. Based on previous studies which determine that the buccal glands (ASG) are mucous in nature the focus of this study is to compare the anterior (buccal glands/ASG) and posterior (esophageal glands/PSG) salivary glands, followed by a comparison of their left and right side and, secondly, an investigation and comparison of the venom segregation of the species living in the southern Pacific Ocean and in the northern China Sea.

Table 1: Toxins segregated by esophageal glands (PSG) and buccal glands (ASG) of octopuses (Fry et al 2009, 2012; Erspamer et al 1953, 1962; Grisley et al 1987, 1993; Ghiretti 1959, 1960; Songdahl 1974; Lo Bianco 1888)			
Components		PSG	ASG
Amines	<i>acetylcholine, histamine, octopamine, serotonin, taurine, tyramine, amino oxidases</i>	YES	NOT KNOWN
Cephalotoxin(s)	<i>“Ghiretti's cephalotoxins” (alpha cephalotoxin, beta cephalotoxin), glycoproteinaceous toxin</i>	YES	NOT KNOWN
Tetrodotoxin		YES	NOT KNOWN
Proteinaceous toxins (Tachykinin peptides)	<i>eledoisin, OctTK 1 & OctTK 2, homologue & 6 novel putative toxins, CriSP, phospholipase A2, E-cephalotoxin, pacifastin preoteinase inhibitors (PPIs), PLA2, CAP</i>	YES	NOT KNOWN
Enzymes	<i>S1 peptidase gene transcripts, hyaluronidase, chitinase, carboxypeptidase, metalloprotease, serine proteases</i>	YES	NOT KNOWN

2. Material and Methods

Venoms, extracted from marine organisms, are heavily contaminated with mucus and difficult to clean. For cleaning *Octopus* venom the process of an "acetone precipitation" has been utilized for the first time to achieve better results regarding the purity of the venom. Previous applications of the acetone precipitation, cleaning venom of fish and stingray species (Fry, 2014), have shown that it is a very effective technique to get rid of more undesirable particles, in particular mucus, than the exclusive use of the centrifuge. The gel images turned out much stronger and clearer.

A number of techniques have been applied, namely the one-dimensional (1D SDS-PAGE) and the two-dimensional (2D SDS-PAGE) sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tiselius, 1937), Liquid chromatography–mass spectrometry (LC/MS), Mass spectrometry (MS) and the “shotgun proteomics” (Wu, 2002).

For separating and visualizing as well as for characterization of protein mixtures and in combination with mass spectrometric identification the 1D and 2D electrophoresis (developed by Arne Tiselius in 1937) are excellent, diagnostically conclusive tools.

Looking at less complex samples and investigating the most abundant proteins, the 1D SDS PAGE is used also for comparing different production batches on a single gel.

2D PAGE is used for separating soluble proteins with isoelectric points from pI 3-10 and Mw from 10-250 kDa. The protein bands of the 1Ds and the protein spots of the 2Ds can be cut out from the gel and identified by a mass spectrometric analysis and database search, providing the protein name and database accession number.

“Shotgun proteomics” allows the ability to systematically profile dynamic proteomes as well as global identification (Wu, 2002, Alves, 2007). The proteome of a cell is very dynamic and dependent on varying environmental conditions and developmental cues in contrast to the (predominantly static) genome of the same cell. To truly understand a protein it has been becoming increasingly clear over the past decades that it is necessary to examine all its interactions. Especially for investigating and understanding cellular processes mediated through protein-protein interactions the “Shotgun proteomics” refers to the direct and rapid analysis of the entire protein complement within a complex protein mixture (Wu, 2002).

Over the last years especially the techniques of the "1D & 2D SDS-PAGE" and the "shotgun proteomics" demonstrated and proved their functionality (Fry, 2013, 2014).

The pedigree was created and its data provided by the Australian Genome Research Facility and the sequenced gene is "MTCOI" (Mitochondrial Cytochrome C Oxidase Subunit I). (Australian Genome Research Facility, 2014)

2.1. Sample collection and preparation

All 13 specimens, 4 from Chile and 9 from Vietnam, were bought in externally good condition at the fish market in Brisbane, deep-frozen and vacuum-packed, transported to the "*Venom lab*" at the University of Queensland, Brisbane, Australia.

The acquisition of specimens from the fish market was a first test of the feasibility of getting specimens in good conditions and well-suited for this kind of analysis. This aspect worked very well and shows that this is an ethical and sustainable way to utilise existing export markets for biological research.

The specimens from Chile belonged to the species *Octopus mimus*, the ones originating from region of Vietnam (suspected *Cistopus taiwanicus*) were suspected to belong to at least two species on the basis of three different gel images between themselves. Because of this uncertainty, DNA-analyses were made for identification by DNA-barcoding.

All specimens were photographed, measured, weighed (**Table 2**) and a tissue sample was taken from the tip of a non-hectocotylized arm. The tissue samples, the posterior salivary glands (esophageal glands/PSG) and the anterior salivary glands (buccal glands/ASG) were collected and put in liquid nitrogen for storage after each gland (left esophageal glands (left PSG) and right esophageal glands (right PSG), left buccal gland (left ASG) and right buccal gland (right ASG) was weighed individually; furthermore



Fig.10: Salivary Glands and buccal mass of *Octopus mimus*.

A-right esophageal gland (right PSG); B-left esophageal gland (left PSG); C-right buccal gland (right ASG), D-left buccal gland (left ASG); E-Buccal mass, F-Beak

the buccal mass was determined (**Table 2,3 & Fig. 10**).

Removal of the esophageal glands (PSG) and buccal glands (ASG) was carried out by a dorsal incision through the mantle immediately posterior to the eyes, revealing the paired (PSG & ASG) glands on either side of the oesophagus (**Fig. 9**); during this procedure the sample was always

kept on ice.

2.2. Protein extraction

Due to the fact that 75% of the specimens from Chile (3 out of every 4) had a missing or incomplete posterior salivary gland (see Table 2,3), the left and right posterior salivary gland of these Chilean specimens were combined to get sufficient quantity of toxins.

After removal and weighing of the glands an extraction buffer (created earlier and kept on ice - see experimental part; containing protease inhibitors) was used for the extraction of proteins from posterior salivary glands (esophageal glands/PSG) and anterior salivary glands (buccal glands/ASG), pooling conspecific extracts (all extraction was done on ice). The tube in which the homogenisation took place was during this process always in contact with ice. Just enough extraction buffer to cover the glands was added before homogenizing the tissue for 20 seconds using a manual tissue homogenizer, followed by a short break just to check whether the homogenisation works. This process was repeated three times. The homogenizer was rinsed first with ethanol, using a small brush (cleaned with ethanol too after each use) followed by rinsing with the remaining buffer. The edge of the homogenizer-tube was as well rinsed off using the same buffer, before allowed to rest on ice for 30 minutes. The extract was then transferred to an Eppendorf tube and centrifuged (Allegra X-22R Centrifuge) at 4 C° for 30 minutes at 14 000 rcf (relative centrifugation force; 14 000 rcf correspond to 13999 G-force/11180 rpm (revolutions per minute)), the resulting supernatant was transferred to a "MILLEX GV Filter Unit 0.45 um Durapore PVDF Membrane". The remaining cells were depolluted and the optical density (OD) of the filtered supernatant was measured by using a "Thermo Scientific Nano Drop 2000" (concentration in mg/mL; wavelength in nm).

2.3. Protein analyses

2.3.1 Acetone precipitation

Additionally Acetone precipitation was performed with the only purpose to separate the toxins from the not requested material in a better way than with the centrifuge. It was never used before processing ocotopod venom but used extensively for fish venom (Fry, 2014).

For the application of an "Acetone precipitation" the assay must be dried down, using a "CentriVap Centrifugal Vacuum Concentrator" for approximately 60 minutes or a

"Laboratory Freeze Dryer" (ALPHA 2- 4 LSC plus), before adding 40 μ l of resuspension buffer "5% ACN (Acetonitrile) and 1% FA (formic acid)". Adequate vortexing is necessary to achieve accurate assay results before adding 360 μ l of Acetone in each tube (nine times as much "5% ACN and 1% FA"). After vortexing each tube again, the assays have to rest over night in the -22 C°-freezer before the acetone can be removed (**Fig. 11**). After drying down the assays once more 50 μ l Milli-Q-water has been added to each tube to resolve the assays.

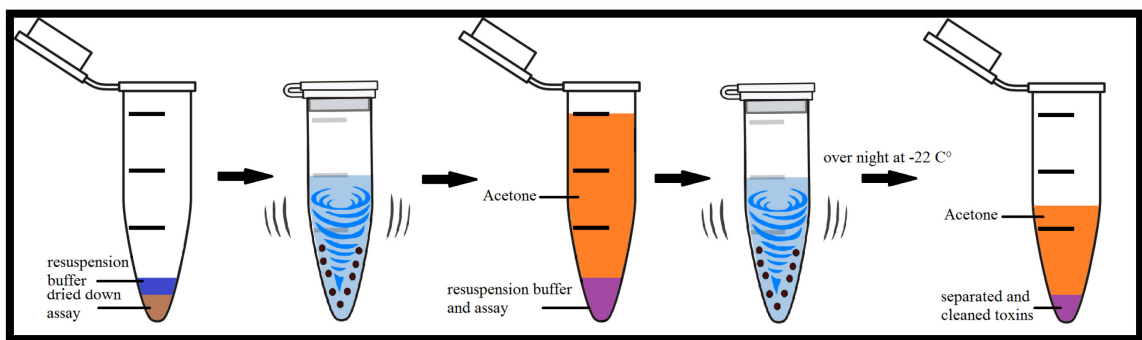


Fig.11: Acetone precipitation - working steps

2.3.2. SDS-Page

Running samples through an 1D (one dimensional) and 2D (two dimensional) Tricine-SDS-PAGE is ideally suited to visualize the contents of the extracts.

A 12% poly-acrylamide gel was used for the "reduced samples" as well as for the "non reduced samples". The extracts were prepared by adding 7.5 μ l dye, containing DTT (Dithiothreitol) for the reduced samples and without DTT for the non reduced samples and by adding Milli-Q-H₂O to adjust every sample to 15 μ l in total. "Reduced" and "non reduced" are the terms used for samples with or without DTT. Adding DTT for the reduced samples was done to break the disulfide bonds, to break up any dimer complexes into individual proteins to get a clearer image.

The venom assays (the ones which were supposed to be reduced, incubated first at 100 C° for 4 minutes in a "thermal cycler" in order to reduce them) were then added to the prepared gel, focused for 15 minutes on 90 V until they reached the resolving gel layer. The voltage was now turned up to 120 V for the reminder of the run for 80 minutes.

The resulting gels were removed afterwards using Milli-Q-H₂O and a scraper, placed in a container and stained in CBB (Coomassie brilliant blue) on a shaker at room temperature.

2.3.3. Highly sensitive Liquid Chromatography combined with Tandem Mass Spectrometry (LC-MS/MS) and the Shotgun Proteomic-analysis

Conducted by the Institute for Molecular Bioscience (IMB) of the University of Queensland the first run of the LC-MS/MS and Shotgun sequencing failed due to a technical breakdown and the second run will be done as soon as possible. Due to time issues the results will be presented in future studies and the upcoming paper.

The highly sensitive "Liquid Chromatography - Tandem Mass Spectrometry" (LC-MS/MS or LC-MS²) is an analytical chemistry technique for separation and identification of molecules by a combination of the liquid chromatography (LC) and the mass spectrometry (MS). (Link, 2009)

The Shotgun Proteomic-analysis (Shotgun Sequencing) is a specific procedure and is used "[...] to identify low molecular weight peptides that do not resolve well on 1D or 2D gels, [...]" (Wu, 2002, Fry, 2014).

2.4. Morphology

This study focuses on the comparison of the salivary glands. Therefore general characteristics of the species as well as information on characters considered relevant to use of venom and prey capture was collected from the literature. Included in the interspecific comparison were the following: size and weight of the buccal glands (ASG) and esophageal glands (PSG), size and weight of the buccal mass (in relation to mantle length) and the length of the specimens in total (**Table 2 & 3**).

Properties of the beak and buccal mass reveal the ability to crush and bite chunks out of the prey while other physical characteristics, except buccal glands (ASG) and esophageal glands (PSG) shape and size, indicates the ability to subdue prey.

3. Results

In this study the first detailed proteomic characterisation of both kinds of salivary glands was done, in form of a comparison of the glands (ASG/PSG) and their left side versus right side using 1D and 2D PAGE, Highly sensitive Liquid Chromatography combined with Tandem Mass Spectrometry (LC-MS/MS) and Shotgun-analyses. In addition the venom segregations of the two analysed species, living in the southern Pacific Ocean and the northern China Sea, were compared.

The results show that gland extracts are a reasonable way to study coleoid venoms though not as ideal as milked venom, it still is useful.

The results of the DNA-analyses (sequenced gen: MTCOI - Mitochondrial Cytochrome C Oxidase Subunit I) show, regarding the species tree, on one hand that in the past some *Cistopus indicus* were mislabeled but provide on the other hand no evidence for subspecies within the clade of *Cistopus taiwanicus* (**Fig. 12**).

All analysed specimen from the northern China Sea are *Cistopus taiwanicus*. The true *Cistopus indicus* is clearly distinct from *Cistopus taiwanicus* (Fry, 2014, 2009). Nonetheless it is indispensable to support these results in following projects and DNA-analyses.

For the South American clade at the bottom of the tree (**Fig. 12**), all specimens are *Octopus mimus*. Whether or not the newly described *Cistopus hubbosorum* as an authentic species is debatable. This has been previously noted (Pliego-Cárdenas, 2014).

3.1. Samples

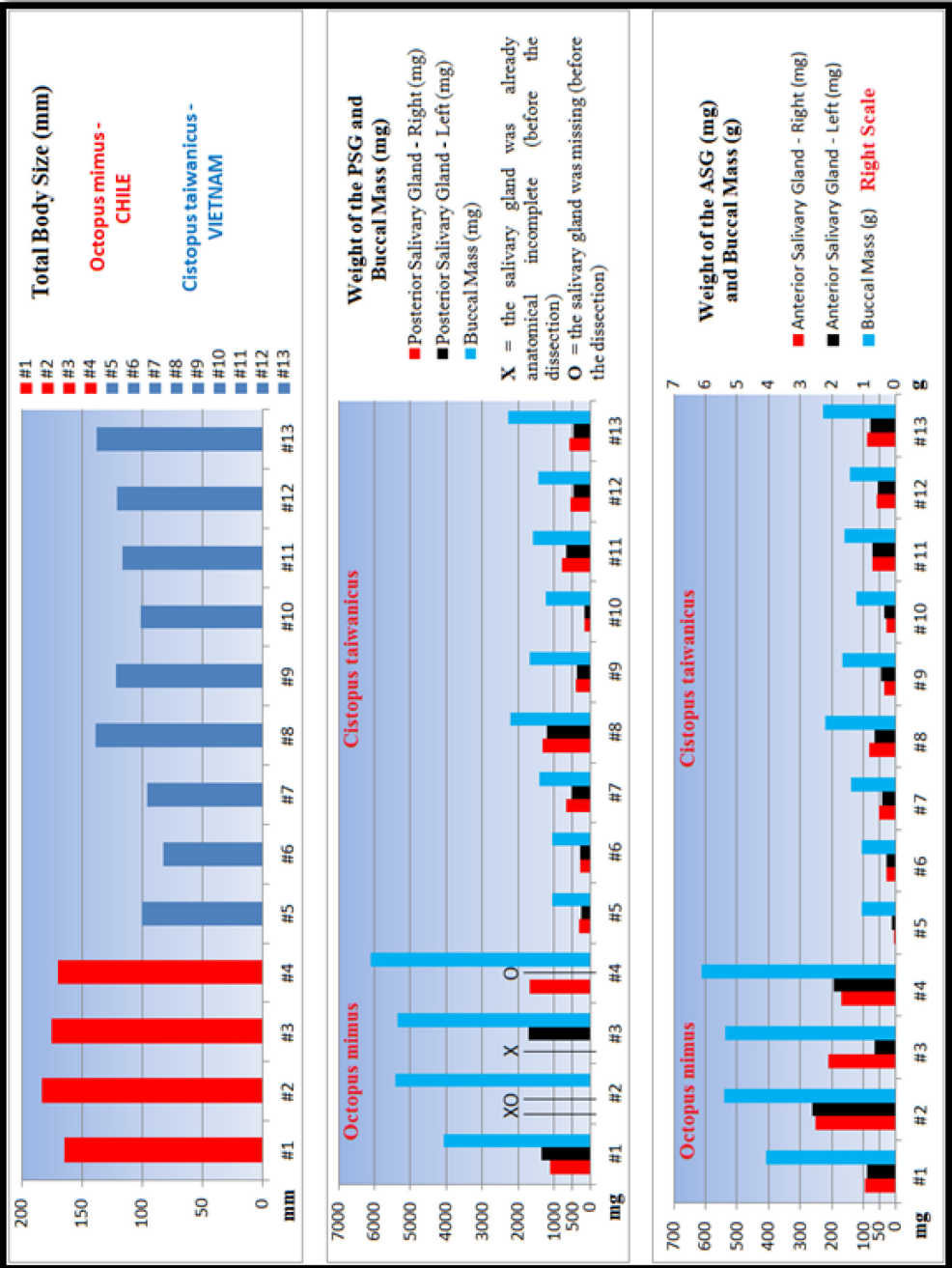
The group of the analysed Octopuses comprised nine specimens from Vietnam and four from Chile, bought in externally good condition at the fish market in Brisbane, deep-frozen and vacuum-packed.

All specimens were photographed, measured, dissected and weighed (**Table 2**). The tissue samples, the posterior salivary glands (esophageal glands/PSG) and the anterior salivary glands (buccal glands/ASG) were collected and put in liquid nitrogen for storage after each gland (left esophageal glands (left PSG) and right esophageal glands (right PSG), left buccal gland (left ASG) and right buccal gland (right ASG) was weighed individually; furthermore the buccal mass was determined (**Table 2,3 & Fig. 10**).

Table 2: A comparison of morphological features, documented after thawing. Abbreviations: TL = Total body length; X = the salivary gland was anatomical incomplete (before the dissection); O = the salivary gland was missing (before the dissection).

Species/Country	Size (max. TL) (mm)	Posterior salivary gland right (mg)	Posterior salivary gland left (mg)	Anterior salivary gland right (mg)	Anterior salivary gland left (mg)	Buccal mass (mg)
<i>Octopus mimus</i> /Chile	165	1122	1352	96.93	87.94	4083
<i>Octopus mimus</i> /Chile	183	X	O	251.87	262.55	5416
<i>Octopus mimus</i> /Chile	175	X	1710	210.84	65.98	5362
<i>Octopus mimus</i> /Chile	170	1688	O	171.29	195.05	6114
<i>Cistopus taiwanicus</i> / <i>Vietnam</i>	100	308	243	2.10	9.44	1055
<i>Cistopus taiwanicus</i> / <i>Vietnam</i>	82	289	282	28.15	28.76	1043
<i>Cistopus taiwanicus</i> / <i>Vietnam</i>	96	663	494	50.07	41.46	1408
<i>Cistopus taiwanicus</i> / <i>Vietnam</i>	139	1331	1189	81.01	64.41	2225
<i>Cistopus taiwanicus</i> / <i>Vietnam</i>	122	391	372	32.90	42.79	1672
<i>Cistopus taiwanicus</i> / <i>Vietnam</i>	101	149	150	27.46	35.54	1226
<i>Cistopus taiwanicus</i> / <i>Vietnam</i>	116	776	652	69.99	70.65	1593
<i>Cistopus taiwanicus</i> / <i>Vietnam</i>	121	554	459	58.09	53.82	1439
<i>Cistopus taiwanicus</i> / <i>Vietnam</i>	138	570	439	90.22	77.71	2269

Table 3: Graphical representation of the body and organ measurements of the four specimens *Octopus mimus* and nine specimens *Cistopus taiwanicus*. Note: Because of the large difference in size and weight, the buccal mass has its own scale at the right side, expressed in grams



3.2. SDS-PAGE

The combined electrophoretic analyses (1D and 2D) revealed without any doubt that there is on one hand a clear difference in segregation between anterior salivary glands and posterior salivary glands (**Fig. 13 & 14**) and on the other hand no difference in segregation between left side and right side (**Fig. 15 & 16**) of each kind of salivary gland (buccal glands/ASG or esophageal glands/PSG) with both *Octopus mimus* and *Cistopus taiwanicus*. As mentioned in the part "protein extraction" the left and right posterior salivary gland of the Chilean specimens were combined to get sufficient quantity of toxins owing to the fact that 75% of the specimens from Chile (3 out of every 4; see **table 2 & 3**) had a missing or incomplete posterior salivary gland. This circumstance may be due to the fact that the Octopuses were bought at the fish market without guarantee of and the possibility to prove the completeness of the specimens.

Furthermore as can be seen from **fig. 17 - 19** there is a clear difference between these two species (*O. mimus* and *C. taiwanicus*). Due to the molecular weights of the already well known components (**table 4**) the proteomics approaches revealed a diverse composition of venom components of both species and both kinds of glands. Focused on the molecular weights the difference between *Octopus mimus* and *Cistopus taiwanicus* is very well perceptible in **figure 19**.

In the further course of the study it could be established that there are differences between some electrophoretic (1D and 2D) images of *Cistopus taiwanicus* (**Fig. 20 - 22**).

By means of the completed 1-Dimensional and 2-Dimensional electrophoretic analyses it could be shown that there is a clear difference between the species *Octopus mimus* and *Cistopus taiwanicus* as well as between the anterior salivary gland (buccal gland/ASG) and posterior salivary gland (esophageal gland/PSG) but no differences between the left and right side of each kind of gland.

The fish market at Brisbane has furthermore turned out as a good opportunity to buy specimens for biological analyses like the SDS-PAGE.

Table 4: Segregated toxins and their approx. molecular weights that are major in venoms of <i>Octopus mimus</i> and <i>Cistopus taiwanicus</i> (Fry 2014)	
Toxin type	Molecular weight (kDa)
Phospholipases A2 (PLA2)	15
CAP	20 - 25
Serine protease	25 - 30
Hyaluronidase	55 - 60
Carboxypeptidase	35 - 50

3.3. Highly sensitive Liquid Chromatography combined with Tandem Mass Spectrometry (LC-MS/MS) and the Shotgun Proteomic-analysis

These methods are a series of very complex analyses and done by the *Institute for Molecular Bioscience (IMB)* of the University of Queensland. The first run failed due to an unpredictable technical breakdown and the second run is repeated at the moment (04/25/2016). Due to time issues the results will be presented in future studies and papers.

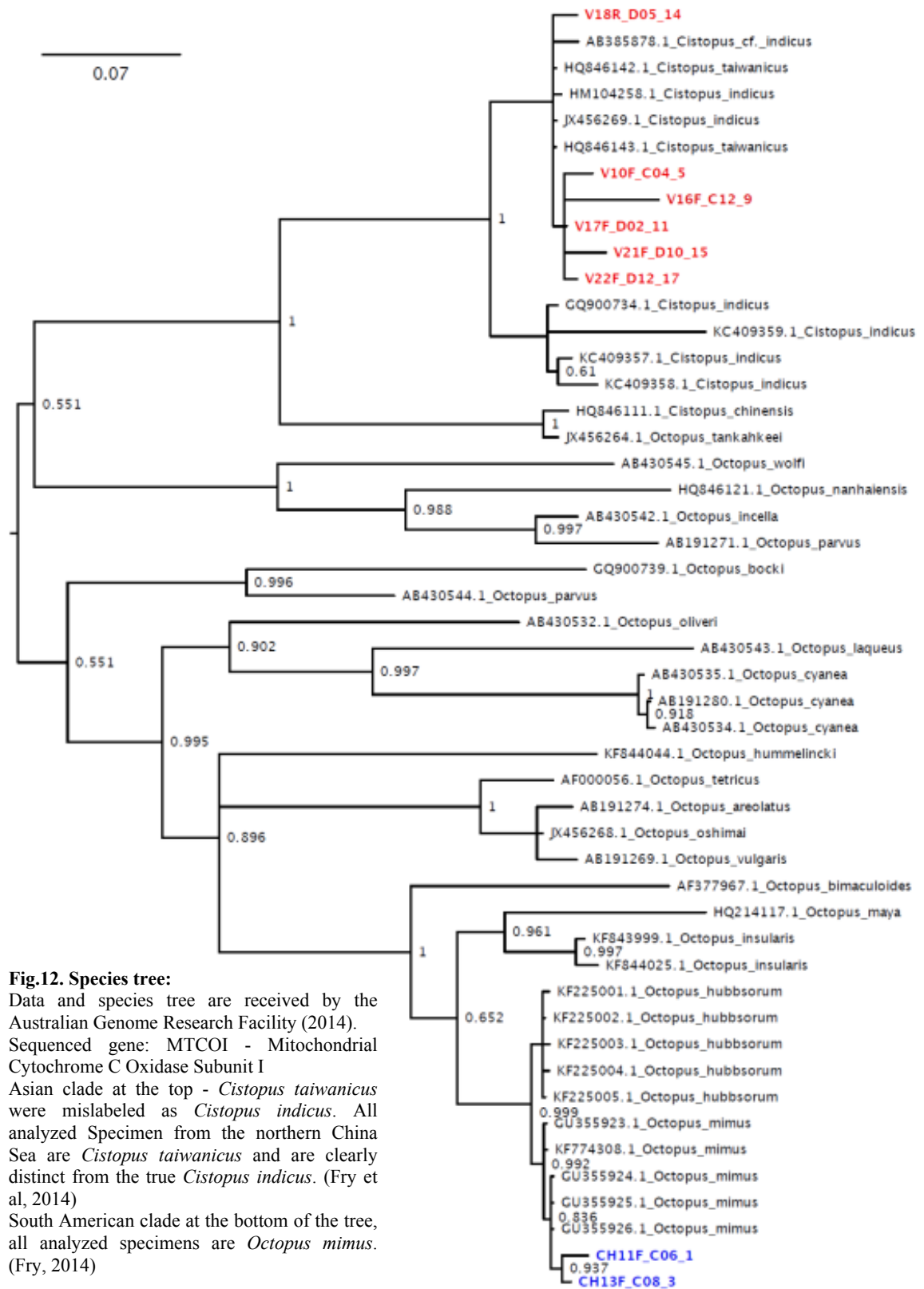


Fig.12. Species tree:

Data and species tree are received by the Australian Genome Research Facility (2014). Sequenced gene: MTCOI - Mitochondrial Cytochrome C Oxidase Subunit I

Asian clade at the top - *Cistopus taiwanicus* were mislabeled as *Cistopus indicus*. All analyzed Specimen from the northern China Sea are *Cistopus taiwanicus* and are clearly distinct from the true *Cistopus indicus*. (Fry et al, 2014)

South American clade at the bottom of the tree, all analyzed specimens are *Octopus mimus*. (Fry, 2014)

CH13F_C08_3 = Fig. 13
V16F_C12_9 = Fig. 14 (no.4)
V18R_D05_14 = Fig. 20 (no.3)
V21F_D10_15 = Fig. 22 (no.5)

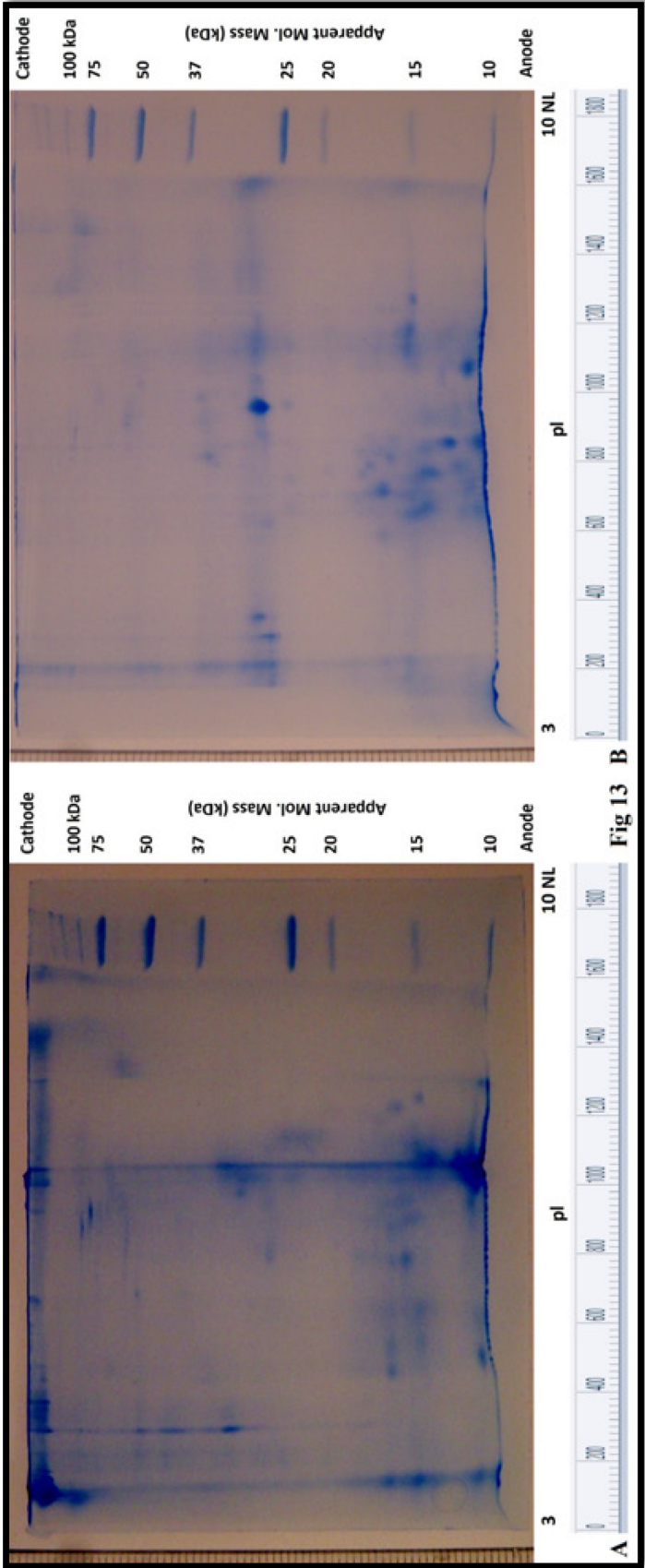


Fig.13. *Octopus mimus*
Fig. 13 and 14 should be used as a comparison between the buccal glands (ASG) and esophageal glands (PSG) of *Octopus mimus* and *Cistopus taiwanicus*.
A: *Octopus mimus*, buccal glands (ASG) **B: *Octopus mimus*, esophageal glands (PSG)**
2-Dimension gel of *Octopus mimus* (CH13F_C08_3) ASG and PSG - venom stained with colloidal coomassie brilliant blue G250. First Dimension: isoelectric focusing (pH3-10 non-linear gradient); Second Dimension: 12% SDS PAGE. The pH gradient and the molecular weight marker positions are shown.

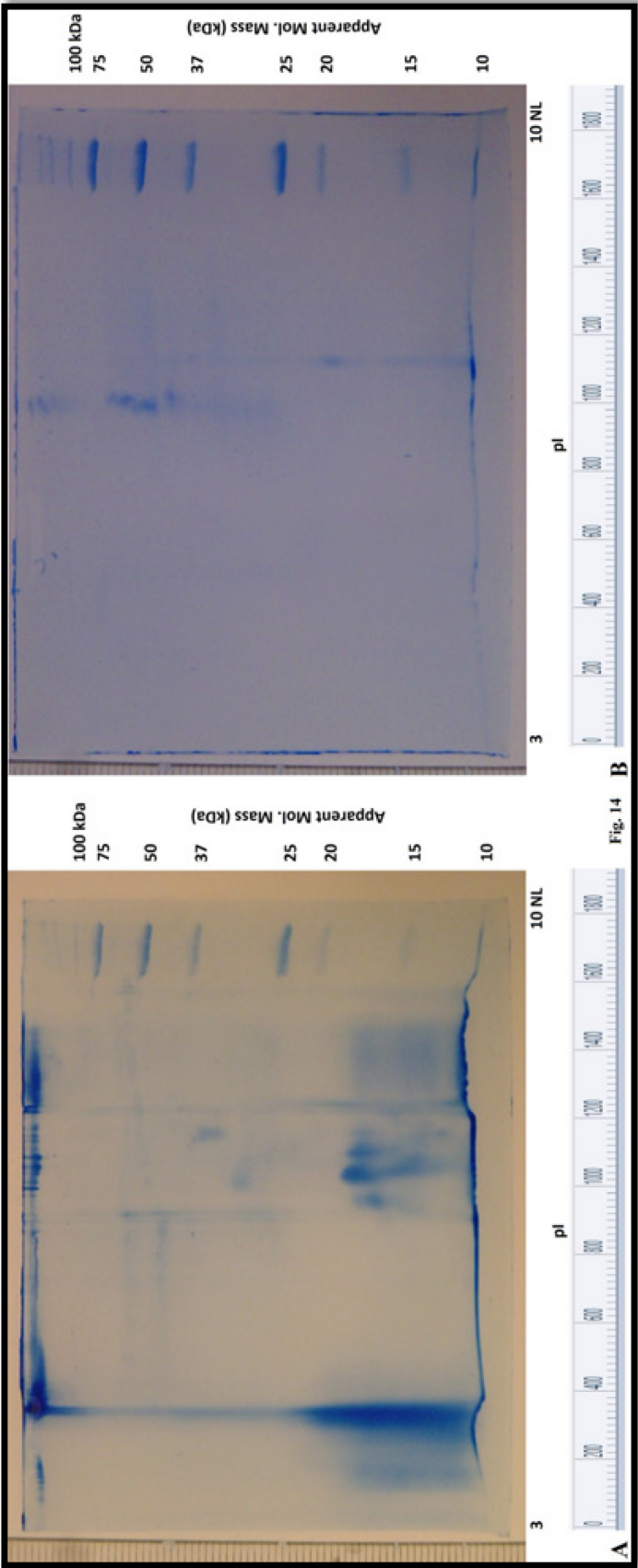


Fig.14. *Cistopus taiwanicus*
Fig. 13 and 14 should be used as a comparison between the buccal glands (ASG) and esophageal glands (PSG) of *Octopus mimus* and *Cistopus taiwanicus*.
A: *Cistopus taiwanicus*, buccal glands (ASG) **B: *Cistopus taiwanicus*, esophageal glands (PSG)**
2-Dimension gel of *Cistopus taiwanicus* (no. 4: V16F_C12_9) ASG and PSG - venom stained with colloidal coomassie brilliant blue G250. First dimension: isoelectric focusing (pH3-10 non-linear gradient); second dimension: 12% SDS PAGE. The pH gradient and the molecular weight marker positions are shown.

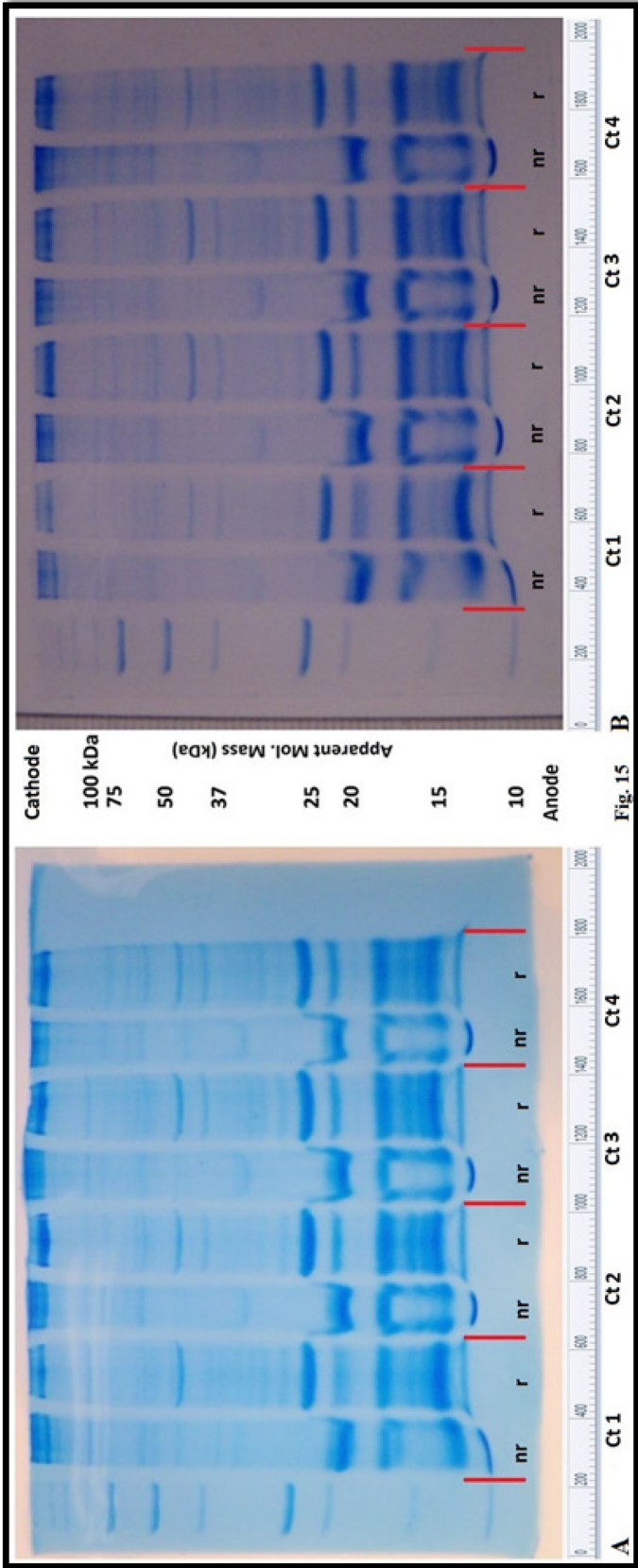


Fig. 15. *Cistopus taiwanicus*
Fig. 15 and 16 should be used as a comparison between the left and right side of the buccal glands (ASG) and esophageal glands (PSG) of *Cistopus taiwanicus*. The 1-Dimensional Gel Electrophoresis was used for the purpose of a better illustration.

A: *Cistopus taiwanicus*, buccal glands (ASG) Left Side
B: *Cistopus taiwanicus*, buccal glands (ASG) Right Side
1-Dimension gel of *Cistopus taiwanicus* ASG- left and right side; 4 specimens: Ct1 = (specimen 1) *Cistopus taiwanicus* 1; nr = non reduced; r = reduced; venom stained with colloidal coomassie brilliant blue G250

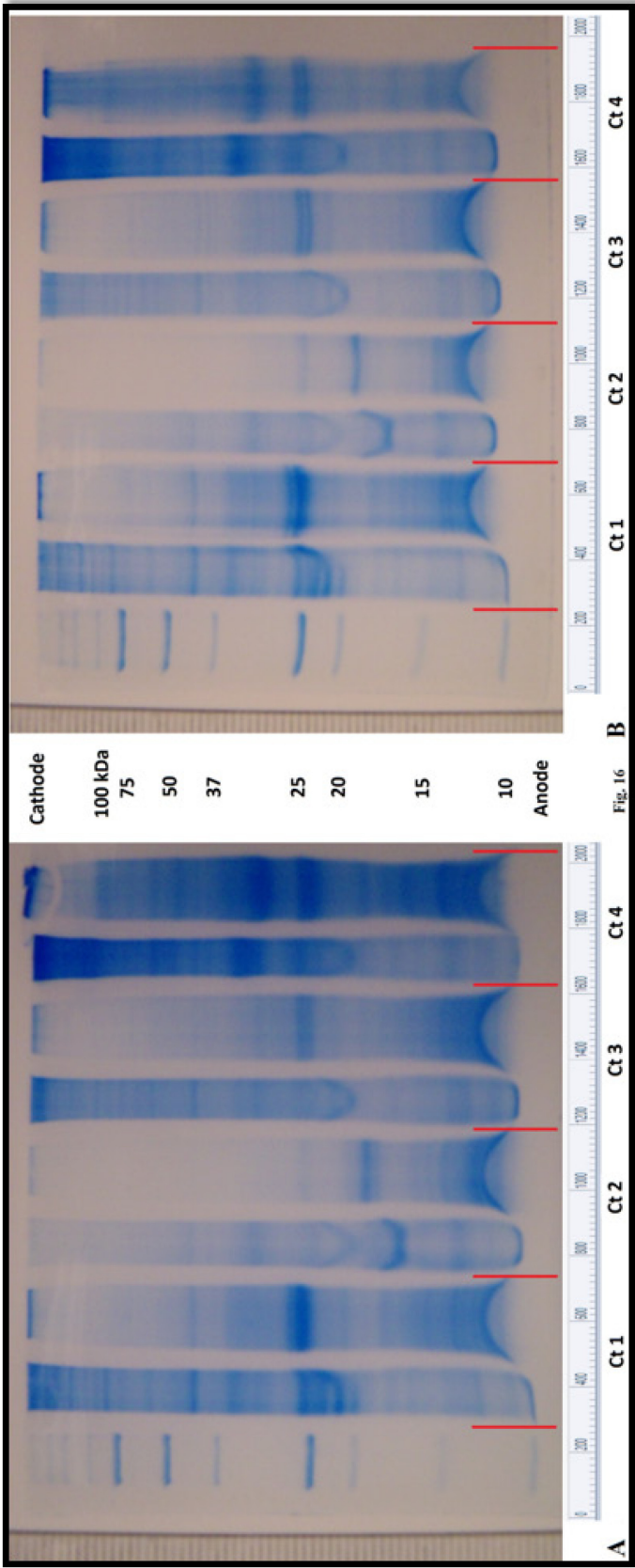


Fig. 16. *Cistopus taiwanicus*
Fig. 15 and 16 should be used as a comparison between the left and right side of the buccal glands (ASG) and esophageal glands (PSG) of *Cistopus taiwanicus*. The 1-Dimensional Gel Electrophoresis was used for the purpose of a better illustration.

A: *Cistopus taiwanicus*, esophageal glands (PSG) Left Side **B: *Cistopus taiwanicus*, esophageal glands (PSG) Right Side**
1-Dimension gel of *Cistopus taiwanicus* PSG left and right side; 4 specimens: Ct1 = (specimen 1) *Cistopus taiwanicus* I; nr = non reduced; r = reduced; venom stained with colloidal coomassie brilliant blue G250

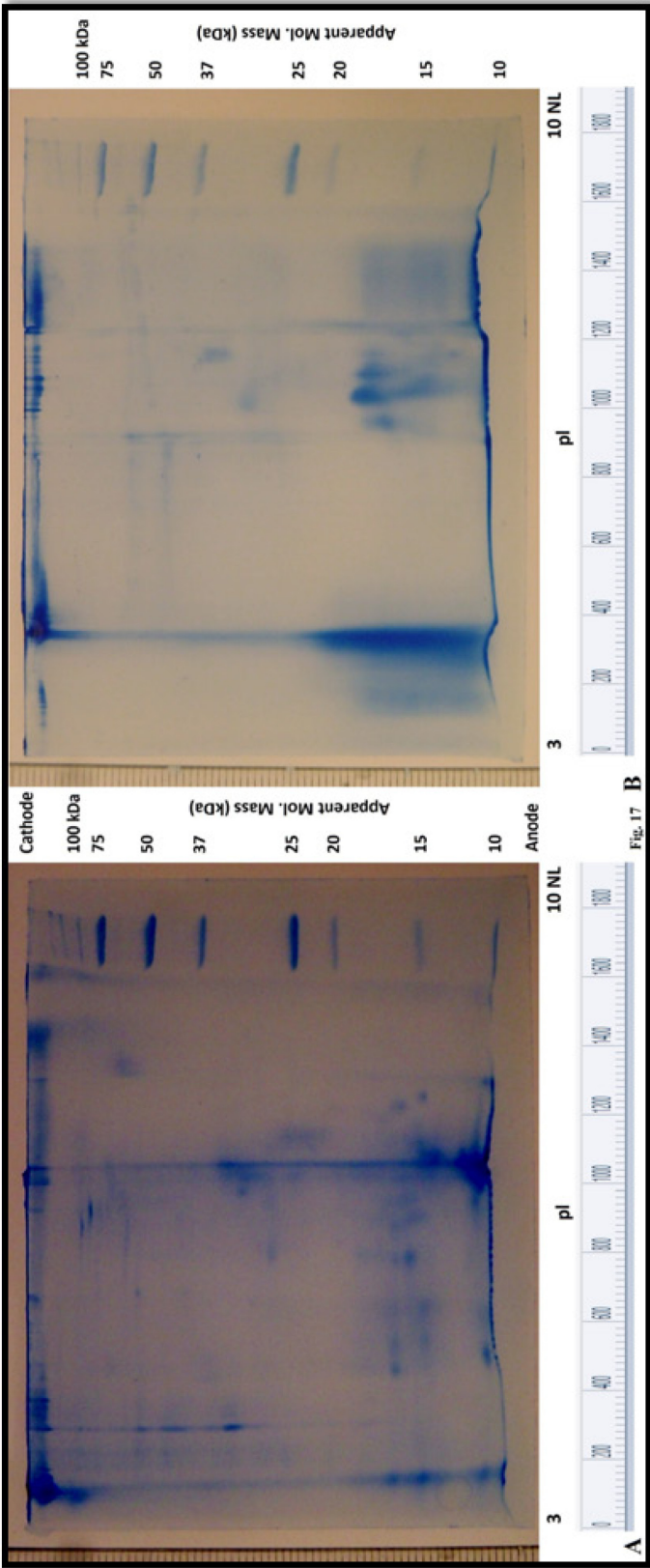


Fig. 17. A *Octopus mimus* and B *Cistopus taiwanicus*
Fig. 17 serves to illustrate the differences between the buccal glands (ASG) of both kinds, *Octopus mimus* and *Cistopus taiwanicus*.

A: *Octopus mimus*, buccal glands (ASG)

B: *Cistopus taiwanicus*, buccal glands (ASG)

2-Dimension gel of the *Octopus mimus* (CH13F_C08_3) ASG and *Cistopus taiwanicus* (no.4; V16F_C12_9) ASG; venom stained with colloidal coomassie brilliant blue G250. First dimension: isoelectric focusing (pH3-10 non-linear gradient); second dimension: 12% SDS PAGE. The pH gradient and the molecular weight marker positions are shown.

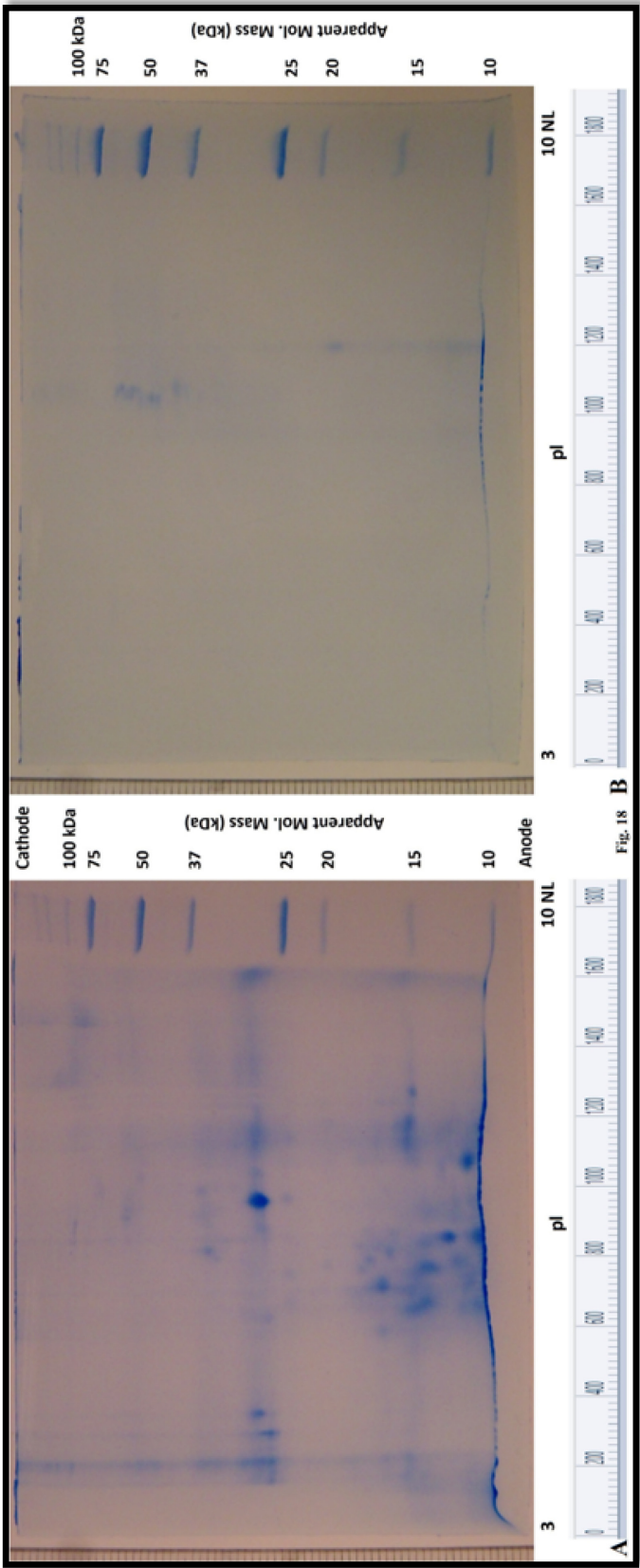


Fig. 18. A *Octopus mimus* and B *Cistopus taiwanicus*
Fig. 18 serves to illustrate the differences between the esophageal glands (PSG) of both kinds, *Octopus mimus* and *Cistopus taiwanicus*.

A: *Octopus mimus*, esophageal glands (PSG)

B: *Cistopus taiwanicus*, esophageal glands (PSG)

2-Dimension gel of the *Octopus mimus* (CH13F_C08_3) PSG and *Cistopus taiwanicus* (no.4; V16F_C12_9) PSG; venom stained with colloidal coomassie brilliant blue G250. First dimension: isoelectric focusing (pH3-10 non-linear gradient); second dimension: 12% SDS PAGE. The pH gradient and the molecular weight marker positions are shown.

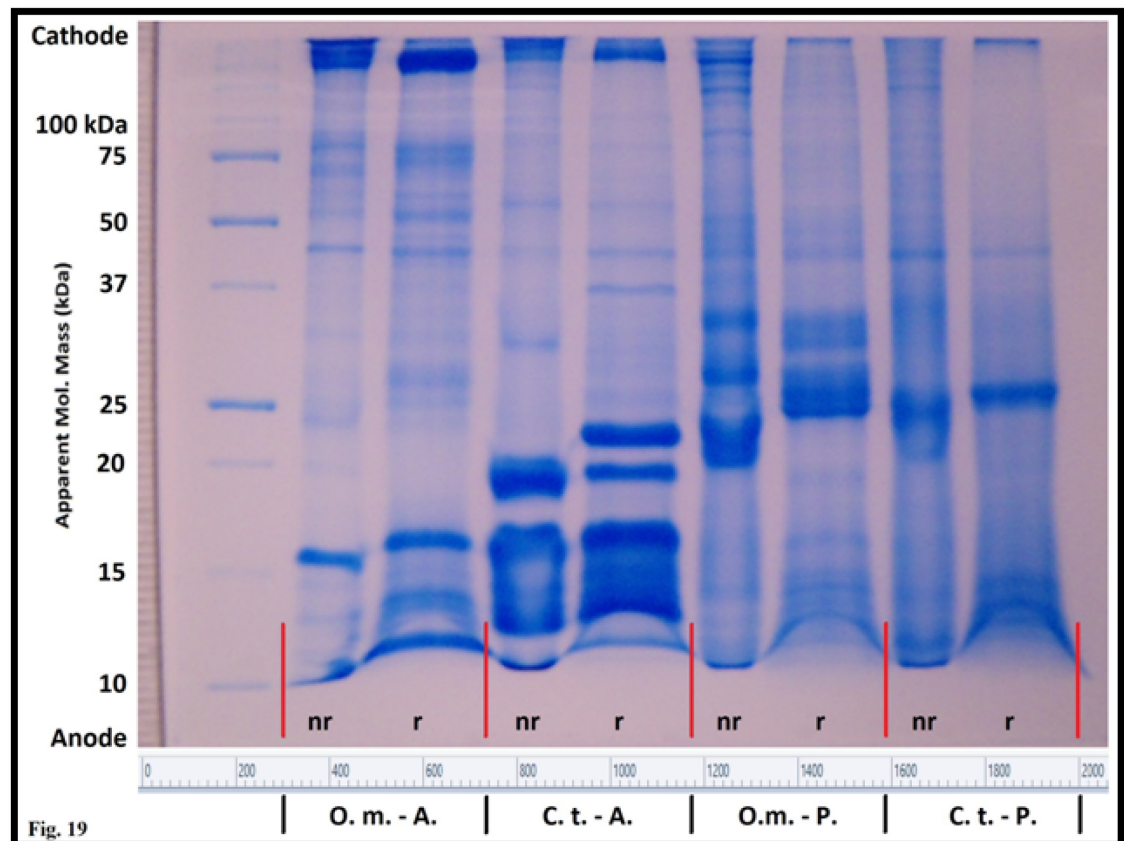


Fig. 19. *Octopus mimus* and *Cistopus taiwanicus*

Fig. 19 should be used as comparison between the buccal glands (ASG) and esophageal glands (PSG) of both kinds, *Octopus mimus* and *Cistopus taiwanicus*. The 1-Dimensional Gel Elektrophoresis was used for the purpose of a better illustration.

1-Dimension gel of *Octopus mimus* (CH13F_C08_3) and *Cistopus taiwanicus* (no. 4; V16F_C12_9); O. m. -A. = *Octopus mimus* Anterior, C. t. -A. = *Cistopus taiwanicus* Anterior, O. m. -P. = *Octopus mimus* Posterior, C. t. -P. = *Cistopus taiwanicus* Posterior, nr = non reduced, r = reduced; venom stained with colloidal coomassie brilliant blue G250

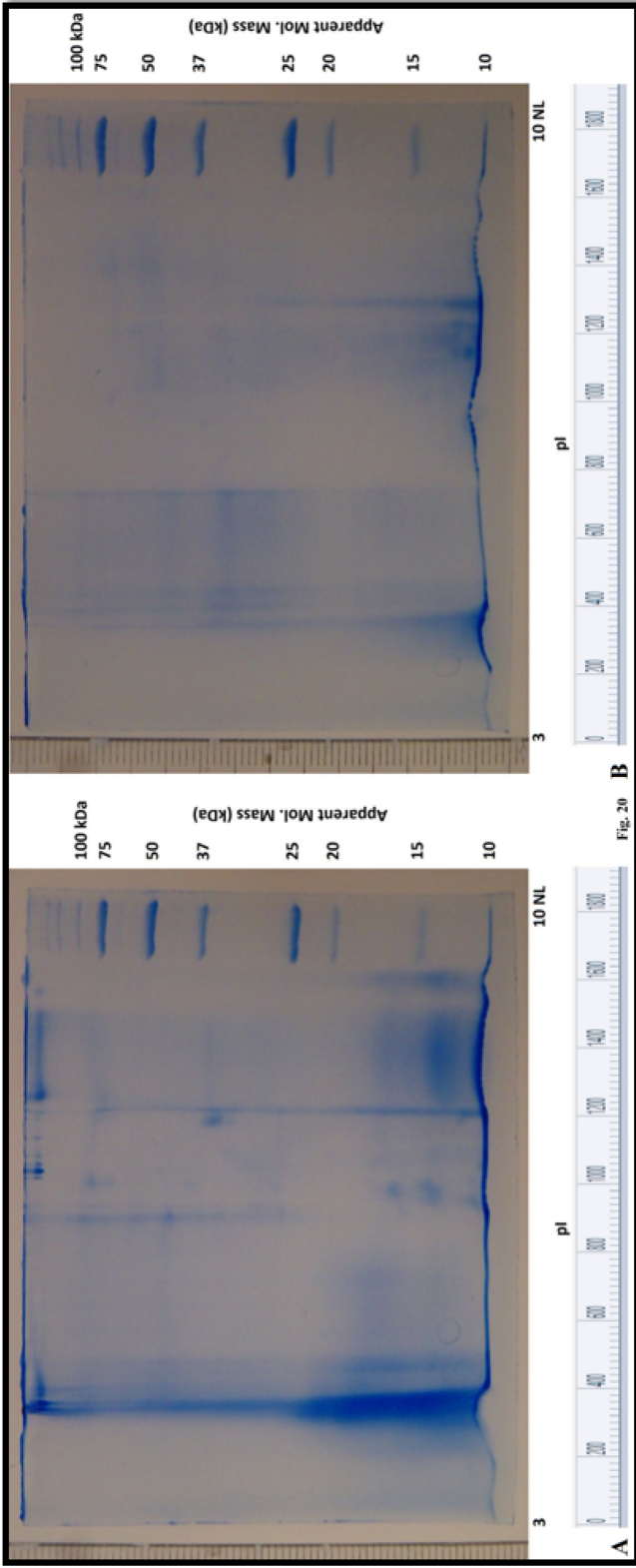


Fig. 20. *Cistopus taiwanicus* (no. 3; V18R_D05_14)
Because of differences between fig. 20 (no. 3; V18R_D05_14), 21 (no. 4; V16F_C12_9) and 22 (no. 5; V21F_D10_15) regarding their gel images and compared to the remaining even gel images of *Cistopus taiwanicus*, fig. 20, 21 and 22 should be used as a comparison between these three specimens, based on assumptions that there could be a subspecies.

A: *Cistopus taiwanicus* (no. 3; V18R_D05_14), buccal glands (ASG) **B: *Cistopus taiwanicus* (no. 3; V18R_D05_14), esophageal glands (PSG)**
2-Dimension gel of the *Cistopus taiwanicus* (no. 3; V18R_D05_14) - obvious differences to the other specimens - venom stained with colloidal coomassie brilliant blue G250. First dimension: isoelectric focusing (pH3-10 non-linear gradient); second dimension: the molecular weight marker positions are shown.

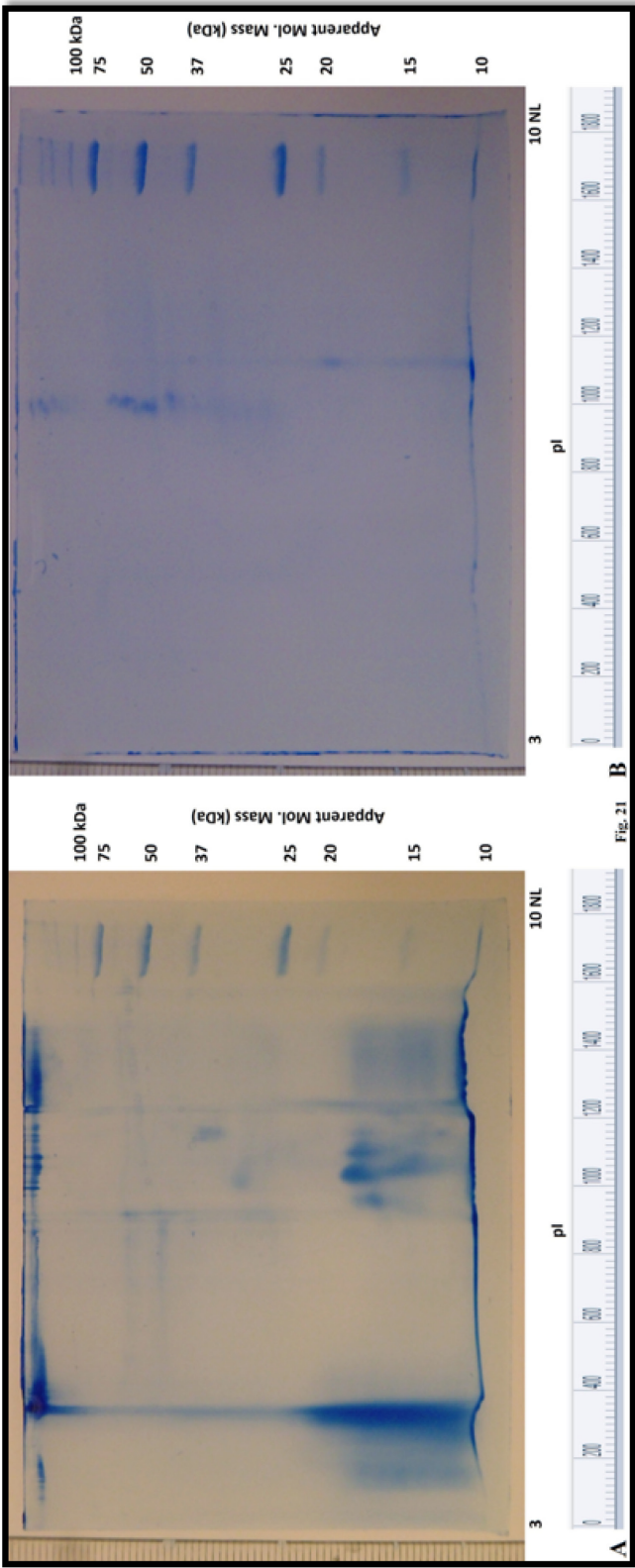


Fig. 21. *Cistopus taiwanicus* (no. 4; V16F C12 9)
Because of differences between fig. 20 (no. 3; V18R_D05_14), 21 (no. 4; V16F_C12_9) and 22 (no. 5; V21F_D10_15) regarding their gel images and compared to the remaining even gel images of *Cistopus taiwanicus*, fig. 20, 21 and 22 should be used as a comparison between these three specimens, based on assumptions that there could be a subspecies.

A: *Cistopus taiwanicus* (no. 4; V16F_C12_9), buccal glands (ASG)

B: *Cistopus taiwanicus* (no. 4; V16F_C12_9), esophageal glands (PSG)

2-Dimension gel of the *Cistopus taiwanicus* (no. 4; V16F_C12_9) - obvious differences to the other specimens - venom stained with colloidal coomassie brilliant blue G250. First dimension: isoelectric focusing (pH3-10 non-linear gradient); second dimension: 12% SDS PAGE. The pI gradient and the molecular weight marker positions are shown.

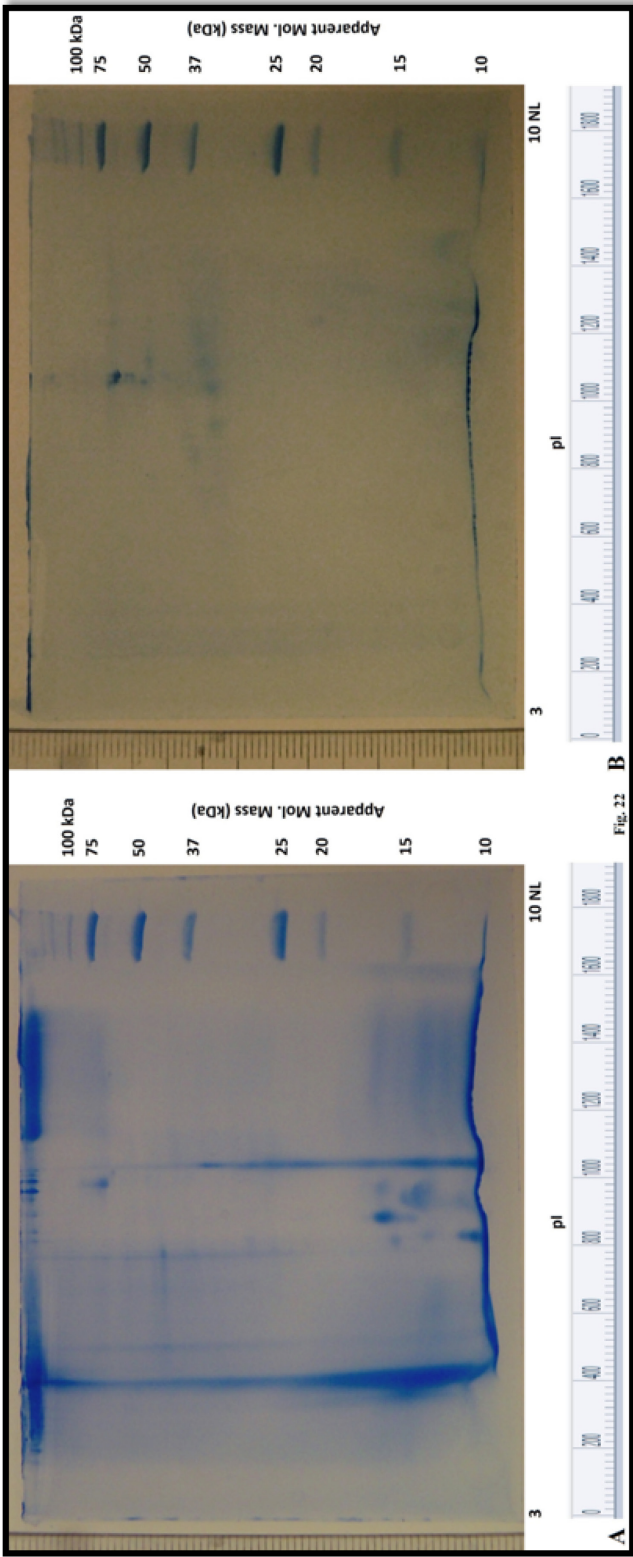


Fig. 22. *Cistopus taiwanicus* (no. 5; V21F_D10_15)
Because of differences between fig. 20 (no. 4; V16F_C12_9) and 22 (no. 5; V21F_D10_15) regarding their gel images and compared to the remaining even gel images of *Cistopus taiwanicus*, fig. 20, 21 and 22 should be used as a comparison between these three specimens, based on assumptions that there could be a subspecies.

A: *Cistopus taiwanicus* (no. 5; V21F_D10_15), buccal glands (ASG) **B: *Cistopus taiwanicus* (no. 5; V21F_D10_15), esophageal glands (PSG)**

2-Dimension gel of the *Cistopus taiwanicus* no. 5; V21F_D10_15, **ASG** - obvious differences to the other specimens - venom stained with colloidal coomassie brilliant blue G250. First dimension: isoelectric focusing (pH3-10 non-linear gradient); second dimension: 12% SDS PAGE. The pH gradient and the molecular weight marker positions are shown.

4. Discussion

Results and influencing factors

This study is a new approach to investigate and analyse the complex venom of Octopods and their segregating gland tissue by comparing the two different kinds of salivary glands (ASG and PSG) as well as their left and right side of each kind of salivary gland.

Two thesis form the crucial point of this study; first, that there is a difference between each kind of salivary gland respectively a difference in their segregations and second, that there is no difference between the left and right side of each kind of salivary gland. In addition DNA- and proteomic-analyses were done to investigate the position of the two kinds of octopuses in the species tree and to take a closer look at the composition of the segregations. The latter did not work because of the circumstances mentioned before.

The results prove the both thesis, shown in the figures 13 - 22. There is to see a clear difference between the buccal glands (ASG) and the esophageal glands (PSG) but no difference between their left and right side. Previous research mainly focused on the considerably larger esophageal glands (PSG) whereby very little is known about the composition of the segregations of the buccal glands (ASG). Further thesis, for example, are dealing with the question whether the segregations of both, esophageal (PSG) and buccal (ASG) glands, mix up to become a specific venom for the Octopus to apply or that components of the buccal glands (ASG) initiate processes within the esophageal glands (PSG). (Fry, 2013)

However, following studies should focus on analyzing the composition of these segregations in order to gain insights into these processes, furthermore because anatomic and external factors affect the composition of these segregations. Factors like kind of prey, intervals between ingestion, age of the specimen, kind of habitat, fitness and the like, can affect the composition of venom and therefore the shape of the gel images of the 1D- and 2D-gel electrophoresis.

The DNA-analyses (sequenced gen: MTCOI - Mitochondrial Cytochrome C Oxidase Subunit I) were done to verify the assumption that, first, in the species tree some *Cistopus indicus* were mislabeled (Fry, 2013) and, second, based on the results of the gel electrophoresis, there are some subspecies within the clade of *Cistopus taiwanicus*.

On one hand the results confirm the assumption that in the past some *Cistopus indicus* were mislabeled but on the other hand provide no evidence for subspecies within the clade of *Cistopus taiwanicus* (Fig. 12).

All analysed specimen from the northern China Sea are *Cistopus taiwanicus*. The true *Cistopus indicus* is clearly distinct from *Cistopus taiwanicus* (Fry, 2014, 2009). Nonetheless it is indispensable to support these results in following projects and DNA-analyses.

Source of supply

For this kind of study the source of supply for the "material" respectively the specimens is always a matter of interest. However, the fish market in Brisbane has furthermore turned out as a good and cheaper opportunity to acquire specimens for biological analyses compared to stay on a fish trawler collecting specimens. Furthermore a fish market provides a wide range of species and the specimens are already deep frozen. But of course there is no guarantee for completeness or accuracy of the specimens anatomy as seen at the specimens *Octopus mimus* from Chile. This also applies for the specimens caught with a trawl (injuries). Due to the tight timetable of the project it was not possible to buy further specimens what should be done in future projects.

Another important aspect for analyzing venom is the food the specimens have ingested and accumulated before. With reasonable certainty food can have an effect on the composition of venom and therefore it is indispensable to analyse the gastric contents. This aspect ought to be the subject of further studies. Another aspect could be the correlation between the body size of the animal and the quantity and quality of the produced venom. Buying the specimens at the fish market is advantageous because of course both, the specimen and its gastric content, are deep frozen. . Octopods are opportunistic feeders, therefore it is possible to find a wide range of food in their stomach.

In conclusion, a fish market is readily a good opportunity to acquire marine specimens for biological analyses.

Techniques

The "Highly sensitive Liquid Chromatography combined with Tandem Mass Spectrometry (LC-MS/MS)" and "Shotgun Sequencing" are well known and frequently used but as their names already imply, very sensitive methods for separation and identification of (low molecular weight) peptides/molecules. It was necessary sending the samples to a specialized and experienced institute. Unfortunately even they are not immune against technical breakdowns. As they receive a lot of samples from all over the world within a short space of time, any problem can cause a massive time lag. Due to the above mentioned technical problems, the tight timetable and the fact that these analyses take a lot of time, unfortunately no results of the "LC-MS/MS" and "Shotgun-analyses" can be presented in this study. Further studies have shown that these analyses are well suited to identify toxins and based on that they have to be repeated in the future.

Another promising technique is the "acetone precipitation" to achieve better results regarding the purity of venom. Previous applications of the acetone precipitation for example for cleaning venom of fish or stingray species (*Fry, 2014*) under comparable conditions, have shown that it is a very effective technique to get rid of more undesirable particles than just using the centrifuge. It was utilized for the first time for cleaning Octopus-venom and it turned out to be a very effective technique to clean marine venoms.

SDS-Page (1D and 2D electrophoresis developed by *Arne Tiselius in 1937*) is a time-tested excellent, diagnostically conclusive analysis for separating and visualizing as well as for characterization of protein mixtures and is part of the standard repertoire to characterize venoms. The results of the SDS-Page analyses confirmed the initial theory that there is a difference between the kinds of salivary glands respectively no difference between right or left side.

5. Summary

The aim of this thesis was first to examine whether there is or is no difference between the segregations of the buccal (anterior) and esophageal (posterior) salivary glands of *Octopus mimus* and *Cistopus taiwanicus* and second whether there is or is no difference between the left and right side of each kind of salivary gland.

Additionally buying specimens for biological analyses at the fish market offered the opportunity to investigate whether a fish market is considered to be a valuable source of supply or not.

The analyses methods used for 13 Octopods (four *Octopus mimus* and nine *Cistopus taiwanicus*) have clearly shown that there is a significant difference between the buccal and esophageal glands but no difference between left and right side.

Furthermore it turned out that fish markets are a convenient and potentially valuable opportunity to receive specimens for biological analyses.

Zusammenfassung

Das Ziel dieser Masters Thesis war, festzustellen ob ein Unterschied zwischen den Segregationsprodukten der Bukkal- (anterior) und Ösophagealdrüsen (posterior) der Arten *Octopus mimus* und *Cistopus taiwanicus* besteht sowie zwischen linker und rechter Seite des jeweiligen Drüsenpaares.

Zusätzlich wurde die Möglichkeit untersucht einen Fischmarkt als Bezugsquelle für Material, welches sich für wissenschaftliche Untersuchungen eignet, zu nutzen.

Die für die 13 untersuchten Exemplare (vier *Octopus mimus* und neun *Cistopus taiwanicus*) verwendeten Analysemethoden haben klar und deutlich gezeigt, dass ein signifikanter Unterschied zwischen den bukkalen und ösophagealen Drüsen besteht, jedoch kein Unterschied zwischen der jeweiligen linken und rechten Seite.

Des Weiteren hat sich herausgestellt, dass ein Fischmarkt eine sehr gut, praktische und wertvolle Möglichkeit darstellt, Material für wissenschaftliche Analysen zu beziehen.

6. List of figures and tables

Figure 1: Scheme of the location and construction of the venom apparatus	8
Figure 2: The venom of a cone snail	9
Figure 3: <i>Nautiloidea</i> - scheme of the inner organization	12
Figure 4: <i>Coleoidea</i> (<i>Sepia officinalis</i>) - scheme of the inner organization	12
Figure 5: Muscles (Nautilus, Squid, Octopus)	15
Figure 6: Mantle structure in squids	16
Figure 7: Radula and jaws	18
Figure 8: Alimentary tract in <i>Coleoidea</i>	19
Figure 9: Dissection of <i>Octopus mimus</i>	20
Figure 10: Salivary glands and buccal mass of <i>Octopus mimus</i>	26
Figure 11: Acetone precipitation - working steps	28
Figure 12: Species tree	35
Figure 13: <i>Octopus mimus</i> - Buccal and Esophageal glands (2D)	36
Figure 14: <i>Cistopus taiwanicus</i> - Buccal and Esophageal glands (2D)	37
Figure 15: <i>Cistopus taiwanicus</i> - Buccal glands left and right side (1D)	38
Figure 16: <i>Cistopus taiwanicus</i> - Esophageal glands left and right side (1D)	39
Figure 17: <i>Octopus mimus</i> and <i>Cistopus taiwanicus</i> - Buccal glands (2D)	40
Figure 18: <i>Octopus mimus</i> and <i>Cistopus taiwanicus</i> - Esophageal glands (2D)	41
Figure 19: <i>Octopus mimus</i> and <i>Cistopus taiwanicus</i> - Buccal and Esophageal glands (1D)	42
Figure 20: <i>Cistopus taiwanicus</i> no. 3 (2D)	43
Figure 21: <i>Cistopus taiwanicus</i> no. 4 (2D)	44
Figure 22: <i>Cistopus taiwanicus</i> no. 5 (2D)	45
 Table 1: Toxins segregated by esophageal and buccal glands	 24
Table 2: A comparison of morphological features	31
Table 3: Graphical representation of the body and organ measurements of the four specimens <i>Octopus mimus</i> and nine specimens <i>Cistopus taiwanicus</i>	32
Table 4: Segregated toxins and their approximately molecular weights that are major in venoms of <i>Octopus mimus</i> and <i>Cistopus taiwanicus</i>	34

EXPERIMENTAL PART

Given to the fact that the majority of the used chemical reagents were self-made but not of necessity following standard protocols it is beneficial to specify the used reagents respectively the used recipes to reconstruct the experimental working steps.

Buffer

Electrophoresis (Running) Buffer 10x pH 8.3 (makes 1 L)

- Tris 30.3 g
- Glycine 144.0 g
- Base SDS 10.0 g
- MQ water 0.75 ml

Extraction Buffer (makes 50 ml)

- Trizma base 300 mg
- MQ water 50 ml (adjust to pH 7.5)
- EDTA 0.185 g
- NaCl 0.292 g
- Np-40 0.5 ml
- PMSF 200 mM 0.25 ml

Gel Buffer (1.5 Mol Tris pH 8.8) makes 500 ml

- 800 ml Jar
- Dissolve 90.75 g Tris base in 400 ml H₂O
- Adjust to pH 8.8 with HCl and make up to 500 ml
- Filter and store at room temperature
- Calibrate the pH-meter first with "pH 4.01"
- Calibrate the pH-meter then with "pH 7.00"
- use: HCl to decrease and 2.5 Mol NaOH to increase the pH

Stacking Buffer

- 70 ml Jar
- Dissolve 6.075 g Tris base
- adjust to 100 ml (MQ)
- Check the pH of the stacking Buffer - has to be 6.8
- Calibrate the pH-meter first with "pH 4.01"
- Calibrate the pH-meter then with "pH 7.00"
- use: HCl to decrease and 2.5 Mol NaOH to increase the pH

Gel overlay Buffer

- Add 1 ml 10% SDS to 25 ml Gel Buffer (1.5 Tris pH 8.8) and dilute to 100 ml (MQ)

Agarose Overlay

- Add 0.5 g low melt agarose to 100 ml gel overlay buffer
- put it into the microwave till it is dissolved
- Add a tiny amount of Bromophenol blue into the liquid agarose

Chemical Reagents

10 % SDS (makes 200 ml)

- 200 ml Jar
- 180 ml MQ
- dissolve 20 g SDS (use the dust mask)

Iso-Butanol

- 200 ml Jar
- MQ 30 ml
- Butanol 70 ml
- mix it under the fume hood and shake it gently

Coomassie

- 800 ml Jar
- Methanol 226 ml
- Phosphoric acid (85%) 24 ml
- Ammonium sulfate 113.3 g
- Coomassie Blue (G-250) 0.6 g
- Adjust with MQ to 600 ml
- stir it over night

Gel Recipes

Resolving Gel

- MQ 3.3 ml
- Tris (pH 8.8) 2.5 ml
- 30 % Acrylamide mix 4.0 ml
- 10 % SDS 100 µl
- 10 % Ammonium sulfate 100 µl
- at the very end: TEMED 4 µl

Stacking Gel

- MQ 1.4 ml
- 30 % Acrylamide mix 330 µl
- Stacking Buffer (pH 6.8) 250 µl
- 10 % SDS 20 µl
- 10 % Ammonium sulfate 20 µl
- at the very end: TEMED 2 µl

Procedures of the 1D and 2D Gel Electrophoresis

1 Dimensional Gel Electrophoresis

Gel preparation

- 1D SDS-PAGE of venom is carried out on 12% polyacrylamide gels
- Resolving gel is made to 10ml (see 5.4.1) and set in a Bio-Rad glass cast for 30 minutes - 6 ml

- H₂O saturated butanol is added to the top of the resolving layer to prevent the gel from drying out 1ml
- Stacking gel is made to 2ml (see 5.4.2) and is added to the cast after the resolving layer had set and the H₂O saturated butanol is removed 1ml
- The 10 well comb is inserted into the stacking layer and was left to set for 20 minutes
- Once the gel was completely set, the comb is carefully removed and the gel caster is assembled. 800ml of 10x electrode buffer is then poured into the container and in-between the caster.

Venom preparation

- 20µg of venom is reconstituted in 10µl MQ-H₂O; 5µl of 3x dye is added bringing the total volume to 15µl
- The venoms are incubated at 100°C for 4 minutes in order to reduce them (just for **REDUCED** samples)

Gel loading and running

- Once the samples had been incubated
 - 4µl of standard protein ladder is pipetted into the first well
 - venom samples are immediately pipetted into the wells in the appropriate order
- Samples are run on 90V until they reached the resolving gel layer; the voltage was then turned up to 120V for the remainder of the run ~ 1.3 h
- The gels are then removed using MQ-H₂O and a scraper and placed in a container
- The gels are placed on an orbital shaker and stained overnight in Coomassie brilliant blue staining

2 Dimensional Gel Electrophoresis

1st Day

- 300µg (in general) freeze-dried venom used
- Add 125µl of solubilization buffer (prepared) to the sample
- Add 0.75µl of Bio-lyte buffer (gently mixing)

- Carefully pipette the appropriate volume of sample as a large drop into the top end of a 50ml test tube - keep it always horizontal.
- Remove the protective layer from the IPG-strip and insert the IPG-strip into the test tube on the top of the sample - gel side down! Avoid any bubbles!
- Seal the test tube with Parafilm and leave the strip to hydrate for a minimum of 8 and a maximum of 24 hours at room temperature.

2nd Day

- Place the hydrated strip gel side up into the strip-holder (first row) ensuring the + or anodic end is at the pointy end of the strip-holder.
- Dampen with 2 pieces of filter paper with distilled water over the ends (without touching the gel) of the IPG-strip.
- Push the electrodes gently down
- Cover the whole row with mineral oil
- Place it on the IPGhor - close the lid (follow the instructions of the IPGhor)

3rd Day

IPG-Strip Equilibration

- IPG strips need to be equilibrated with SDS-Page buffer. Reduction and alkylation of the proteins is done at the same time.
- Take the 5 ml Equilibration-buffer and divide the buffer in half
- Reducing Buffer: add 150 mg DTT per 10ml to one half and for the
- Alkylation Buffer: 200mg iodoacetamide per 10ml to the other half

Calculation:

1st Tube: 2.5ml EQ-Buffer + 150mg DTT/10ml

$$150\text{mg DTT per } 10\text{ml} \rightarrow 4 \times 2.5 = 10\text{ml} \rightarrow 150:4 = \boxed{37.5\text{mg DTT}}$$

~ 15 - 20 minutes

2nd Tube: 2.5ml EQ-Buffer + 200mg iodoacetamide/10ml

$$200:4 = \boxed{50\text{mg iodoacetamide}}$$

~ 30 minutes

- Equilibration Buffer can be prepared in advance and stored frozen but DTT and iodoacetamide must be added fresh
- Put the IPG-strip into the DTT-tube and onto the "multi-rotator" for ~ 15 minutes

- Afterwards put the IPG-strip into the iodoacetamide-tube for ~ 30 minutes
- Prepare the gel
- Remove the isobuthanol and put the Overlay-Buffer in for ~5 minutes
- Remove the Overlay-Buffer and add agarose on top of the gel
- Insert the equilibrated strip through the agarose so it sits directly on top of the resolving gel. Ensure no bubbles are trapped between the strip and the gel!
- Allow agarose to set (fridge)
- Run the gel with 20A for the whole run

REFERENCES

- [1] ALVES P., ARNOLD J. R., NOVOTNY M. V., RADIVOJAC P., REILLY J. P., TANG H. (2007): *"Advancement in protein inference from shotgun proteomics using peptide detectability"*, Pacific Symposium on Biocomputing 12:409-420 (2007)
- [2] BIDDER A. M., (1966), chap. 3: *"Feeding and digestion in Cephalopods"* IN: "Physiology of Mollusca", Volume 2, p. 97-124, Eds. Wilbur K.M., Young C.M. Academic Press, New York & London, ISBN: 978-1-4832-3242-3
- [3] BOYD R. K., (1994): *"Linked-scan techniques for MS/MS using tandem-in-space instruments"*, Mass Spectrometry Reviews 13 (5-6): 359-410. doi:10.1002/mas.1280130502.
- [4] BOYER L. V., THEODOROU A. A., BERG R. A., MALLIE J., CHAVEZ-MENDEZ A., GARCIA-UBBELOHDE W., HARDIMAN S., ALAGON A., (2009): *"Antivenom for critically ill children with neurotoxicity from scorpion stings"*, The New England Journal of Medicine 360, 2090e2098.,
- [5] CAVAZZONI E., LISTER B., SARGENT P., SCHIBLER A., (2008): *"Blue-ringed octopus (Hapalochlaena sp.) envenomation of a 4-year-old boy: a case report."*, Clinical Toxicology 46, 760-761; ISSN: 1556-3650 (Print) 1556-9519; DOI: 10.1080/15563650701601790
- [6] COLE T. C. H., HAUSSER-SILLER I., (2014): *"Dictionary of Biology: English/German"* (4th edition), Springer Berlin Heidelberg; Auflage: 4 (24. November 2014), ASIN: B00R3G9OUQ
- [7] CORTEZ T., CASTRO G. B., GUERRA A., (1998): *"Drilling behaviour of Octopus mimus Gould"*, Journal of Experimental Marine Biology and Ecology, vol. 224, p. 193-203
- [8] DUDA T. F., PALUMBI S. R., (1999): *"Molecular genetics of ecological diversification: duplication and rapid evolution of toxin genes of the venomous gastropod Conus"*, Proc. Natl. Acad. Sci. U.S.A. Vol. 96, pp. 6820-6823
- [9] ERSPAMER V., ASERO B., (1953): *"Isolation of Enteramine from extracts of posterior salivary glands of Octopus vulgaris and of Discoglossus pictus skin"*, The Journal of Biological Chemistry, Vol. 200, p. 311-318.
- [10] ERSPAMER V., ANASTASI A., (1962): *"Structure and pharmacological actions of eledoisin, the active endecapeptide of the posterior salivary glands of Eledone."*, Cell. Mol. Life Sci. 18, 58-59.
- [11] FIORITO G., GHERARDI F., (1999): *"Prey-handling behaviour of Octopus vulgaris (Mollusca, Cephalopoda) on bivalve preys"*, Behavioural Processes. 46: 75-88
- [12] FRY B.G., VIDAL N., VAN DER WEERD L., KOCHVA E., CAMILA R. (2009): *"Evolution and diversification of the Toxicofera reptile venom system"*, Journal of Proteomics Vol. 72, p. 127-136;

- [13] **FRY B.G., ROELANTS K., NORMAN J.A., (2009):** *"Tentacles of venom: toxic protein convergence in the Kingdom Animalia."*, Journal of Molecular Evolution 68, 311–321; DOI 10.1007/s00239-009-9223-8;
- [14] **FRY B.G., ROELANTS K., CHAMPAGNE D.E., SCHEIB H., TYNDALL J.D.A., KING G.F., NEVALAINEN T.J., NORMAN J.A., LEWIS R.J., NORTON R.S., RENJIFO C., RODRIGUEZ DE LA VEGA R.C., (2009):** *"The Toxicogenomic Multiverse: Convergent Recruitment of Proteins Into Animal Venoms"*, The Annual Review of Genomics and Human Genetics, doi: 10.1146/annurev.genom.9.081307.164356
- [15] **FRY B.G., UNDHEIM E.A.B.; GEORGIEVA D.N., THEON H.H., NORMAN J.A., MORK J., BETZEL C., (2010):** *"Venom on ice: First insights into Antarctic octopus venoms"*, Toxicon vol. 56, p. 897-913, doi:10.1016/j.toxicon.2010.06.013
- [16] **FRY B.G., CASEWELL N. R., WÜSTER W., VONK F., HARRISON R. A., (2012):** *"Complex cocktails: the evolutionary novelty of venoms"*; Cel Press - Trends in Ecology and Evolution, Vol. 28, No. 4
- [17] **FRY B.G., LOW DOLYCE H.W., SUNAGAR K., UNDHEIM EIVIND A.B., ALI SYED A., ALEJANDRO C. A., TIM R., JACKSON T.N.W., PINEDA GONZALEZ S., KING G.F., JONES A., ANTUNES A., (2013):** *"Dracula's children: Molecular evolution of vampire bat venom"*; Journal of Proteomics 89, 95-111
- [18] **FRY B.G., ALI SYED A., KATE B., TIMOTHY T.N.W., KELLY W., STEVEN M., UNDHEIM E.A.B., NOUWENS A., KOLUDAROV I., HENDRIKX I., JONES A., (2013):** *"Proteomic comparison of Hypnale hypnale (Hump-Nosed Pit-Viper) and Calloselasma rhodostoma (Malayan Pit-Viper) venoms"*; Journal of Proteomics 91, pp. 338-343
- [19] **FRY B.G., ALI SYED A., YANG D.C., JACKSON T.N.W., UNDHEIM E.A.B., KOLUDAROV I., WOOD K., JONES A., HODGSON W.C., MCCARTHY S., RUDER T., (2013):** *"Venom proteomic characterization and relative antivenom neutralization of two medically important Pakistani elapid snakes (Bungarus sindanus and Naja naja)"*; Journal of Proteomics 89, pp. 15-23
- [20] **FRY B.G., SUNAGAR K., UNDHEIM E.A.B., SCHEIB H., GREN E.C.K., COCHRAN C., PERSON C.E., KOLUDAROV I., KELLN W., HAYES W.K., KING G.F., ANTUNES A., (2014):** *"Intraspecific venom variation in the medically significant Southern Pacific Rattlesnake (Crotalus oreganus helleri): Biodiscovery, clinical and evolutionary implications"*, Journal of Proteomics Vol. 99, p. 68-83;
- [21] **FRY B. G., KOLUDAROV I., JACKSON T.N.W., SUNAGAR K., NOUWENS A., HENDRIKX I., (2014):** *"Fossilized Venom: The Unusually Conserved Venom Profiles of Heloderma Species (Beaded Lizards and Gila Monsters)"*, Toxins 2014, 6, 3582-3595; doi:10.3390/toxins6123582

- [22] **FRY B.G., BAUMANN K., CASEWELL N.R., SYED A. A., JACKSON T.N.W., VETTER I., DOBSON J.S., CUTMORE S.C., NOUWENS A., LAVERGNE V. (2014):** *"A ray of venom: combined proteomic and transcriptomic investigation of fish venom composition using barb tissue from the blue-spotted stingray (Neotrygon kuhlii)"*; Journal of Proteomics 109, 188-198

- [23] **FRY B. G., (2015):** *"Venomous Reptiles and Their Toxins: Evolution, Pathophysiology, and Biodiscovery"*, Oxford University Press Inc (13. Mai 2015), ISBN-10: 0199309396, ISBN-13: 978-0199309399

- [24] **GENNARO J.F., LORINCZ JR. A.E., BREWSTER H.B., (1965):** *"The anterior salivary gland of the Octopus (octopus vulgaris) and its mucous secretion"*, Annals of New York Academy of Sciences, Vol. 118, Mucus in Invertebrates - Collected paper, p. 1021-1025, Nov. 1965

- [25] **GHIRETTI F., (1959):** *"Cephalotoxin: the Crab-paralysing Agent of the Posterior Salivary Glands of Cephalopods"*, Nature 183, 1192-1193, doi:10.1038/1831192b0

- [26] **GHIRETTI F., (1960):** *"Toxicity of octopus saliva against crustacea"*, Annals New York Academy of Sciences, p. 726-741

- [27] **GRISLEY M.S., BOYLE P.R., (1987):** *"Bioassay and proteolytic activity of digestive enzymes from octopus saliva"*, Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, Volume 88B, no. 4, pp. 1117-1123

- [28] **GRISLEY M.S., (1993):** *"Separation and partial characterization of salivary enzymes expressed during prey handling in the octopus Eledone cirrhosa."*, Comp. Biochem. Physiol. B. Biochem. Mol. Biol., Vol. 105B, pp. 183–192.

- [29] **GRUBERT M.A., WADLEY V.A., WHITE R.W.G., (1999):** *"Diet and feeding strategy of Octopus maorum in southeast Tasmania"*, Bulletin of Marine Science, Vol. 65 (No.2): p. 441–451,

- [30] **KANDA A., IWAKOSHI-UKENA E., TAKUWA-KURODA K., MINAKATA H., (2003):** *"Isolation and characterization of novel tachykinins from the posterior salivary gland of the common octopus Octopus vulgaris"*, Peptides 24, 35–43

- [31] **KYRIACOU A., AHMED A.B., (2010):** *"Exenatide Use in the Management of Type 2 Diabetes Mellitus"*, Pharmaceuticals 3, doi:10.3390/ph3082554

- [32] **LEWIS R.J., GARCIA M.L., (2003):** *"Therapeutic potential of venom peptides."*, Nat. Rev. Drug Discov. 2, 790–802, doi:10.1038/nrd1197

- [33] **LINK A.J., LABEAR J., (2009):** *"Proteomics: A Cold Spring Harbor Laboratory Course Manual"*, Cold Spring Harbor Laboratory Press; 1st edition (2008-12-12) (1656), ASIN: B019L4QYPK

- [34] **LO BIANCO S., (1888):** "Notizie biologiche riguardanti specialmente il periodo di maturità sessuale degli animali del golfo di Napoli", Mitt. zool. Stn. Neapel 19: 385-440 (Italian)
- [35] **LO BIANCO S., (1888):** "Notizie biologiche riguardanti specialmente il periodo di maturità sessuale degli animali del golfo di Napoli", Mitt. zool. Stn. Neapel 19: 513-761 (German)
- [36] **MAGGENTI M.A., MAGGENTI A., GARDNER S., (2011):** "Dictionary of Invertebrate Zoology", Zea E-Books, ISBN-10: 1609620011, ISBN-13: 978-1609620011
- [37] **MCDONALD N.M., COTTRELL G.A., (1972):** "Purification and mode of action of toxin from *Eledone cirrhosa*", Toxicon, Vol. 8, issue: 2, p. 142, ISSN: 0041-0101, DOI: 10.1016/0010-4035(72)90033-X
- [38] **MEBS, D., (2002):** "Venomous and Poisonous animals: A Handbook for Biologists, Toxicologists and Toxinologists, Physicians and Pharmacists", "Wissenschaftliche Verlagsgesellschaft"; Auflage: 1 (1. April 2002) 1., 1 - 25, 70 76, 182, 251 - 330., ISBN-10: 3804750230, ISBN-13: 978-3804750234
- [39] **MORISHITA T., (1974):** "Participation in digestion by the proteolytic enzymes of the posterior salivary gland in *Octopus-II* Isolation and purification of the proteolytic enzymes from the posterior salivary gland.", Bulletin of the Japanese Society of Scientific Fisheries; Nippon Suisan Gakkai Shi 40, 601–607.
- [40] **NIXON M.,(1984):** "Is there external digestion by *Octopus*?", Journal Zoology 202, 441–447.
- [41] **PILSON M.E.Q., TAYLOR P.B., (1961):** "Hole drilling by octopus", Science 134, 1366–1368.
- [42] **PLIEGO-CÁRDENAS R., HOCHBERG F.G., DE LEÓN J.G., DE LOS ANGELES BARRIGA-SOSA I., (2014):** "Close Genetic Relationships between Two American Octopuses: *Octopus hubbsorum* Berry, 1953, and *Octopus mimus* Gould, 1852", Journal of Shellfish Research, vol. 33(1), p. 293-303, DOI: <http://dx.doi.org/10.2983/035.033.0128>, URL: <http://www.bioone.org/doi/full/10.2983/035.033.0128>
- [43] **RUNHAM N.W., BAILEY C.J., CARR M., EVANS C.A., MALHAM S., (1997):** "Hole drilling in crab and gastropod shells by *Eledone cirrhosa* (Lamarck, 1798)", Sci. Mar. 61, 67–76
- [44] **SALVINI-PLAWEN L.L.v., (1988),** chap.: "A reconsideration of systematics in the Mollusca (Phylogeny and higher classification)" IN: "Malacologia", Volume 19, p. 249–278.
- [45] **SALVINI-PLAWEN L.L.v., (1988),** chap.: "The Structure and Function of Molluscan Digestive Systems" IN: "The Mollusca - Form and Function", Volume 11 (Wilbur K.M.), p. 301-374, Academic Press Inc., San Diego, New York, Berkeley Boston, London, Sydney, Tokyo, Toronto
- [46] **SAPORITO R.A., DONNELLY M.A., JAIN P., GARRAFFO H. M., SPANDE T.F., DALY J.W., (2007):** "Spatial and temporal patterns of alkaloid variation in the poison frog *Oophaga pumilio* in Costa Rica and Panama over 30 years".; Toxicon 50, pp. 757-778

- [47] **SHEUMACK D.D., HOWDEN M.E.H., SPENCE I., QUINN R.J., (1978):** "*Maculotoxin: a neurotoxin from the venom glands of the octopus *Hapalochlaena maculosa* identified as tetrodotoxin*", Science Vol. 199, pp. 188–189.
- [48] **SHIGENO S., SASAKI T., MORITAKI T., KASUGAI T., VECCHIONE M., AGATA K., (2008):** "*Evolution of the cephalopod head complex by assembly of multiple molluscan body parts: evidence from *Nautilus* embryonic development.*", Journal of Morphology, Vol. 269, pp.1-17
- [49] **SINGER B.J., SILBERT B.I., SILBERT P.L. SINGER K.P., (2015):** "*The Role of Botulinum Toxin Type A in the Clinical Management of Refractory Anterior Knee Pain*", Toxins 7, doi:10.3390/toxins7093388
- [50] **SOLEM. A., ROPER C.F.E., (1975):** "*Structures of Recent Cephalopod Radulae*", The Veliger Vol. 18, no 2, page 127-133
- [51] **SONGDAHL J.H., SHAPIRO B.I., (1974):** "*Purification and composition of a toxin from the posterior salivary gland of *Octopus dofleini**", Toxicon Vol. 12, pp. 109–115
- [52] **STEER M.A., SEMMENS J.M., (2003):** "*Pulling or drilling, does size or species matter? An experimental study of prey handling in *Octopus dierythraeus* (Norman, 1992)*", Journal of Experimental Marine Biology and Ecology, 290 (2003) 165– 178
- [53] **STORCH V., WELSCH U., (2002):** "*Kükenthals Leitfaden für das Zoologische Praktikum*", Spektrum Akademischer Verlag; Auflage: 24. Aufl. (22. August 2002), ISBN-10: 3827411114, ISBN-13: 978-3827411112
- [54] **STRUGNELL J., NORMAN M., JACKSON J., DRUMMOND, ALEXEI J., COOPER A., (2005):** "*Molecular phylogeny of coleoid cephalopods (Mollusca: Cephalopoda) using a multigene approach; the effect of data partitioning on resolving phylogenies in a Bayesian framework.*", Molecular Phylogenetics Evolution, Vol. 37, pp. 426–441
- [55] **TERLAU H., OLIVERA B.M., (2004):** "*Conus Venoms: A Rich Source of Novel Ion Channel-Targeted Peptides*", the American Physiological Society, Physiol Rev. Vol. 84
- [56] **TISELIUS A., (1937):** "*A new apparatus for electrophoretic analyses of colloidal mixtures*", Trans. Faraday Soc., 1937,33, 524-531, DOI: 10.1039/TF9373300524
- [57] **TSETLIN V.I., (2014):** "*Three-finger snake neurotoxins and Ly6 proteins targeting nicotinic acetylcholine receptors: pharmacological tools and endogenous modulators*", CellPress, Trends in Pharmacological Sciences, Vol. 36, No. 2
- [58] **WALKER P., (1999):** "*Chambers Dictionary of Science and Technology*", Chambers; Auflage: Revised edition (6. August 1999), ISBN-10: 0550141103, ISBN-13: 978-0550141101

- [59] **WELLS M.J., (1988)**, chap.: *"The Mantle Muscle and Mantle Cavity of Cephalopods"* IN: *"The Mollusca - Form and Function"*, Volume 11 (Wilbur K.M.), p. 287 - 299, Academic Press Inc., San Diego, New York, Berkeley Boston, London, Sydney, Tokyo, Toronto
- [60] **WESTHEIDE W., RIEGER R., (2007)**: *"Spezielle Zoologie Teil 1: Einzeller und Wirbellose Tiere"*, IV.8, 352 - 361., Springer Spektrum; Auflage: 3., überarb. u. aktual. Aufl. 2013 (11. Juli 2013), ISBN-10: 3642346952, ISBN-13: 978-3642346958
- [61] **WILBUR K.M., (1988)**: *"The Mollusca - Form and Function"*, Volume 11, Academic Press Inc., San Diego, New York, Berkeley Boston, London, Sydney, Tokyo, Toronto
- [62] **WU C.C, MACCOSS M.J., (2002)**: *"Shotgun proteomics: Tools for the analysis of complex biological systems"*, Current Opinion in Molecular Therapeutics (2002) 4(3):242-250
- [63] **YOTSU-YAMASHITA M., MEBS D., FLACHSENBERGER W., (2007)**: *"Distribution of tetrodotoxin in the body of the blue-ringed octopus (Hapalochlaena maculosa)."*, Toxicon 49, pp. 410–412
- [64] <http://tolweb.org/tree?group=Cephalopoda>, (May, 2015)
- [65] <http://tolweb.org/onlinecontributors/app?service=page/ScientificContributorsPage>), (May, 2015) *"Tree of Life Scientific Contributors"*
- [66] <http://www.venomdoc.com/venomdoc/Coleoids.html>, (June, 2015)
- [67] **"CephBase"**, (April - June, 2015), <http://web.archive.org/web/20050211122343/www.cephbase.utmb.edu/>
- [68] ["http://www.mdpi.com/journal/toxins"](http://www.mdpi.com/journal/toxins), (June, 2015)
- [69] ["http://www.mdpi.com/2072-6651/7/9/3388"](http://www.mdpi.com/2072-6651/7/9/3388), (July, 2015)

ACKNOWLEDGMENT

At the end of my master's thesis I want to express my particular gratitude towards Associate Professor Dr. Bryan Fry for giving me the chance to be part of a great experience and a professional team of his "Venom Laboratory" at the University of Queensland (Australia), associate Professor Dr. Gerhard Steiner (University of Vienna) for his invaluable support, experience and patience writing this masters and M.Sc. James Dobson, a very promising and talented young scientist for his support during work in the laboratory. They were always ready to listen if I had open questions and find a way out of complex issues.

My special thanks count for Prof. Dr. phil. nat. Dietrich Mebs (Goethe-University, Frankfurt; treasurer of the International Society on Toxinology and Jean Servais Stas-medal winner) who gave me his permission using the amazing graphical material of his books and supported me with interesting and inspiring conversations.

My wife Manuela Meirer for her patience and support, proofreading and creative help.

My brother, assistant Professor Dr. Florian Meirer (University of Utrecht) who helped me with words and deeds at any time.

My parents, who believed in me and generously provided me with the foundation for everything I managed to achieve in my life and for everything I have become.