

Chapter 1

Introduction

1.1 Snake Venom

1.1.1 General Information

The production of venom, with a highly efficient mechanism for its delivery, allows for successful restraint of prey, which Russell (1980) and Savitzky (1980) suggest as being responsible for venomous snakes becoming the most highly developed of all reptiles.

Of the more than 3500 species of snakes that exist today less than 400 are known to be venomous (Russell and Brodie, 1974). Families and examples of modern venomous snakes are as follows (Rabb and Marx, 1973; White, 1981):

1. Viperidae

- (a) Viperinae - puff adders, Gabon vipers, Russell's vipers, horned vipers, saw-scaled vipers and European vipers.
- (b) Crotalinae - rattlesnakes, wateroccasins, copperheads, bushmasters and certain primitive pitvipers of Asia.

2. Elapidae - cobras, tiger snakes, coral snakes, mambas and kraits.

3. Hydrophiidae - sea snakes.

4. **Colubridae** - mostly non-venomous snakes, but a few such as the boomslang and some other rear-fanged tree snakes of Africa are venomous.

This study is mainly concerned with the Hydrophiidae with some references to Elapidae due to their close affinities.

During its 20 million years of evolution, snake venom, along with the fangs and venom glands, according to Russell and Brodie (1974), has obtained a high degree of development.

The venom glands of snakes are thought to have evolved from oral glands, homologous with parotid glands (Porter, 1972; Sutherland, 1983). They are ovate in shape, situated on either side of the head behind and below the eyes (Worrel, 1967; Halstead, 1970; Gans, 1978; White, 1981) and are enveloped by a tough fibrous capsule, from which fibres of the mandibular muscle arise (Sutherland, 1983). It is this muscle that is responsible for gland compression forcing venom out and through the adjoining fang (deVries and Condrea, 1971).

In the hydrophiids, the venom glands are of the elapid type (Rosenberg, 1967; Barme, 1968; Kochva and Gans, 1971; Minton, 1974; Minton, 1980), possessing an anterior mucus gland and a posterior main gland (glandula venefica). The main gland consists of parallel tubules lined with secretory epithelium, which empty into the main duct that continues as the duct of the anterior gland. Secretory granules accumulate in the epithelial cells and venom is stored in the tubular lamina. In the elapid-type venom gland, space for venom storage, according to Kochva and Gans (1971) and Minton (1974), is reduced due to the lack of a central lumen such as is found in the Viperidae. This explains the relatively low venom yields in hydrophiids and elapids as compared to vipers. According to Rosenberg (1967), similarities do exist among these taxa regarding the general features of the secretory process.

The fangs, probably initially functioning in the holding of prey (Gans, 1978) and / or the deep penetration of salivary fluids (Kardong, 1979; Heatwole, 1987), became modified as an apparatus injecting venom into the tissue of an intended prey. Fang type can be used as a basis for one type of classification of venomous snakes as follows (Porter, 1972; Smith *et al.*, 1977):

1. **Opisthoglyphs** - snakes with rear fangs.

2. **Solenoglyphs** - snakes with movable front fangs.

3. **Proteroglyphs** - snakes with non-movable front fangs.

Hydrophiids and elapids are of the proteroglyph type (Porter, 1972; deVries and Condrea, 1971), possessing rigidly fixed, short, front fangs in an erect position (Minton, 1974; Sutherland, 1983), and located on the anterior part of the maxilla (Barne, 1968).

The fangs of hydrophiids are folded to form an open groove in some cases or a tube that is completely closed off in others (Halstead, 1970; Porter, 1972; Minton, 1980; White, 1981). The groove is still visible on the anterior face of the latter (deVries and Condrea, 1971). The relatively large venom duct of hydrophiids empties into the base of the fang.

Venom was thought to have first evolved as lubricant assisting swallowing of prey (Barne, 1968; Minton, 1974; Gans, 1978; Kochva, 1978). This was necessary, according to Kochva (1978), since reptiles' primitive life styles offered them only dry prey items that were difficult to swallow.

Quite by accident, sometime later, according to Minton and Minton (1980), some salivary enzymes proved to be toxic and promoted internal digestion when introduced into the victim's tissue. With natural selection favouring the production of these substances, prey immobilization (Minton, 1974; Minton and daCosta, 1975; Heatwole, 1977) and facilitation of internal digestion (Kochva and Gans, 1971; Heatwole, 1977; Gans, 1978; Minton and Minton, 1980; Russell, 1980; Gans, 1983) resulted. Kochva and Gans (1970) point out that many authors consider prey immobilization the most important of the two.

Prey immobilization is thought to be important for snakes, because of their carnivorous habits, feeding mainly on living animals, which according to Minton (1974), can cause problems for the snake. With large struggling prey, the potential problem faced by snakes are damage to the fragile jaws and soft buccal cavity, risk of the prey escaping and other injuries to the snake through defensive attacks by the prey (Gans, 1978; Sutherland, 1983; Heatwole, 1987). The ability to subdue prey quickly would help in minimizing such risks through decreasing the prey's activity during capture.

In accordance with their limblessness, snakes have evolved backwardly curved teeth that help prevent prey from escaping (Romer, 1959; Heatwole, 1977). According to Heatwole (1977), these teeth types are useless for the mechanical breakdown (cutting, tearing and grinding) of tissue, and along with the lack of other similarly functioning external structures (i.e. claws and beaks), snakes have retained the habit of their ancestral lizards of swallowing whole prey (Gans, 1961). Through the introduction of digestive enzymes deep into the prey, the problem of digesting large prey would be minimized (Gans, 1961; Kochva and Gans, 1970; Kochva and Gans, 1971; Russell, 1980). The prey can be digested from the inside out as well as from outside inward, preventing putrefaction from occurring (Kochva *et al.*, 1983).

The defensive function of the venom, according to Schmitd (1950), Barne (1968), Heatwole, (1977) and Gans, (1978), is thought to be a secondary development in the evolution of most snake venoms.

Venoms are complex mixtures of materials containing many component parts (Worrel, 1967; Russell and Brodie, 1974; Mebs, 1978; Heatwole, 1987). Reptile venoms, according to Minton and Minton (1980), possess numerous large, active complex molecules, most of which are difficult to analyze. Snake venoms have multiple roles (Russell and Brodie, 1974; Heatwole, 1977) and are surpassed by no other biological toxins in their biological and pharmacological complexity (Minton, 1974). Minton (1974) further points out that their complexity arises from snakes adding new components to their previous arsenal during evolution.

A large proportion of snake venom, approximately 90 to 95% according to Barne (1968) and Tu (1977), consists of noncellular proteins. This group contains the major and minor toxins, small peptides and nontoxic proteins with no known biological activities (Barne, 1968) and enzymes. Total toxicity of the venom is the result of the synergistic effects of all toxic components (Barne, 1968; Chang, 1979; Heatwole, 1987), with the activities of venoms varying from species to species, due to different protein constituents (Barne, 1968).

The nonprotein portion of the venom is divided into organic and inorganic parts (Tu, 1977). The organic part consists of free amino acids, nucleotides and related compounds, carbohydrates, lipids and biogenic amines. The inorganic components

are various anions and cations.

Snake venoms are known to contain at least twenty-six different enzymes (Minton and Minton, 1980; White, 1981), with about half of them found in most snake venoms, but with no species containing them all. This production of numerous enzymes with a diversity of biological activities allows for venomous snakes to be far more versatile than their non-venomous counterparts (Russell and Brodie, 1974), in terms of capture of prey. Some of the more important of these enzymes are as follows:

1. The phospholipase A group is one of the most widespread of the venom enzymes (Minton, 1974), and is found in most snake venoms. It possesses little affinity for erythrocytes, attacks membrane phospholipids, both cellular and intracellular (Minton and Minton, 1980), disrupts electron transport as well as mitochondrial integrity and has known myocytic properties causing skeletal muscle and kidney damage (Barne, 1968; Fohlman and Eaker, 1977; Tu, 1977).
2. Proteases are enzymes responsible for breaking down proteins; they disrupt blood clotting, damage capillaries causing hemorrhage and cause tissue death by liquifying actions (Minton and Minton, 1980).
3. Hyaluronidase enzyme has little toxic effect, but promotes the spread of other venom components by dissolving the gel surrounding the cells, thereby facilitating the movement of the components through the tissue (Barne, 1968; Minton and Minton, 1980).
4. Other enzymes are involved more with digestion than toxicity *per se*. For example nucleotidases break down phosphorous-containing compounds in the cell nuclei.

Most of the lethal effects of snake venom appears to arise from polypeptides and proteins of low molecular weights (Minton, 1974; Russell and Brodie, 1974; Habermehl, 1981), rather than from enzymes.

1.1.2 Hydrophiid venom

Due to the close affinities between the elapids and hydrophiids (Romer, 1959; Romer, 1967; Barne, 1968; deVreis and Condrea, 1971; Lee, 1971a; Russell and Puffer, 1971; Minton and daCosta, 1975; Mao *et al.*, 1977; Tu, 1977; Minton and Minton, 1980; Mao *et al.*, 1983) in phylogeny, morphology, anatomy and venom (apparatus, chemistry and action), elapid studies have contributed to a better understanding of the hydrophiids. Hydrophiids and their venoms have not been extensively investigated compared to other venomous snakes, due to difficulties in their capture and their low yields of venom. Much more work is needed to be carried out on this group.

Sea snake venom is considered rather primitive, according to Tu (1977), due to its simple composition with fewer enzymes than terrestrial snakes (Reid, 1975). A summary of the known makeup of hydrophiid venom is as follows (Russell and Puffer, 1971; Russell and Brodie, 1974; Fohlman and Eaker, 1977; Tu, 1977; Minton and Minton, 1980; Habermehl, 1981):

1. Phospholipase A causing extensive skeletal muscle and kidney damage.
2. Hyaluronidase promoting the spreading of other venom components.
3. Digestive enzymes.
4. Proteolytic enzymes are absent (or present in only small quantities).
5. Lethal polypeptides and low molecular weight proteins (neurotoxins and myotoxins).

1.1.3 Neurotoxic components

It has long been established that elapid and hydrophiid venoms possess toxic components that affect the nervous system (Halstead, 1970; Datyner and Gage, 1973; Maeda and Tamiya, 1974; Chang, 1979; White, 1981). The most characteristic signs for this type of envenomation is the paralysis of muscles serving the respiratory system, resulting in cessation of ventilation (Carey and Wright, 1961; Su *et al.*, 1967; Pickwell, 1972; Tu, 1977; Chang, 1979; Lee and Lee, 1979). The first muscles affected

are those served by the cranial nerves involved in swallowing and eye movement, with the ventilatory muscles affected last (Campbell, 1979; Chang, 1979). Evidence supports neurotoxins as being responsible for this toxicity, through peripheral nerve and neuromuscular transmission impairment (Kellaway *et al.*, 1932; Gitter and deVries, 1967; Barme, 1968; Chang, 1979).

The major group of neurotoxins in elapids and hydrophiids act upon the neuromuscular junctions by irreversibly binding to the cholinergic acetylcholine (ACh) receptors of the postsynaptic membranes (Carey and Wright, 1961; Lee and Tseng, 1966; Tu, 1967; Barme, 1968; Chotia, 1970; Kochva and Gans, 1971; Pickwell, 1972; Prives *et al.*, 1972; Fulpis *et al.*, 1973; Moody *et al.*, 1973; Strydom, 1973b; del Castillo and Anderson, 1974; Minton, 1974; Tamiya, 1975; Tu, 1977; Mebs, 1978; Chang, 1979; Minton and Minton, 1980; Sutherland, 1983). This has been supported through radio isotope studies (Sumyk and Hawrylewicz, 1963; Lee and Tseng, 1966; Sato *et al.*, 1970; Weber and Changeux, 1974), as well as electron microscopic autoradiography (Fertuck and Salpeter, 1974). No ultrastructural damage has been observed in these areas, however, as the result of postsynaptic toxins (Lee, 1971a).

The area of the neuromuscular junction where the nerve fibre converges with the skeletal muscle (Florey, 1966; Harder, 1975) is referred to as the synapse. When the nerve impulse arrives at the presynaptic site of the neuromuscular junction ACh is released (Livett, 1976). When the ACh crosses the synapse it binds with the ACh receptor (DeRobertis, 1964; Peper and McMahan, 1972; Porter and Barnard, 1975) in the muscle plasma membrane (postsynaptic site), causing a depolarization of the muscle membrane and a subsequent muscle contraction (Section 1.2.4). The ACh is immediately hydrolyzed by acetylcholine esterase freeing the ACh receptor for future bindings with ACh (Hoyle, 1957; Florey, 1966; Hyden, 1967; Drachman, 1971; Prosser, 1973; Harder, 1975; Tu, 1977; Stein, 1981; Spence and Mason, 1987). When the neurotoxin irreversibly binds with the ACh receptor (non-depolarizing), ACh is prevented from combining with the receptor, thus preventing muscle contraction and leading to eventual ventilatory cessation (Tu, 1977; Karlsson, 1979). This neurotoxic block, according to Tu (1977) and Mebs (1978), does not affect muscle excitability, nerve action potential or ACh release; only the muscle membrane depolarization is

prevented.

Elapid and hydrophiid neurotoxins are said to be curare-like in action (Tamiya *et al.*, 1967; Barne, 1968; Lee, 1970; Lee, 1971b; Tu, 1977; Mebs, 1978; Chang, 1979; Lee and Lee, 1979), which according to del Castillo and Anderson (1974), refers to any substance that blocks neuromuscular transmission without impairing the ability for nerve conduction or muscle contraction. Signs for curare poisoning are similar to those for elapid and hydrophiid envenomation, and also lead to ventilatory failure.

Neurotoxins are divided into the following groups, based on their molecular make-ups (Strydom, 1973b; Tu, 1977; Chang, 1979).

Type I : short-chain neurotoxins with 60 - 62 amino acid residues and four disulfide cross-links holding peptides in an active configuration.

Type II : long-chain neurotoxins with 71 - 74 amino acid residues and five disulfide cross-links holding peptides in an active configuration.

Elapid and hydrophiid venoms possess mostly short chain neurotoxins, which are very similar in their structure (Reid, 1975; Tamiya, 1975; Tu, 1977), further supporting claims of affinities between the two groups. Toxicities, according to Tu (1977), are thought to be due to the arrangement of certain functional groups within the neurotoxin molecule.

Three neurotoxins have been isolated from *Aipysurus laevis* venom, A, B and C, representing 22, 33 and 21% respectively of the total protein in the crude venom, accounting for most of the toxicity of this venom (Maeda and Tamiya, 1976).

More recently, Tremeau *et al.* (1986) have isolated a monoclonal immunoglobulin, which recognizes the ACh receptor binding site of elapid and hydrophiid short-chain neurotoxins, with epitopes being identified through immunological crossreaction experiments (Grognet *et al.*, 1986).

1.1.4 Myotoxic components

Myotoxic components have been previously reported in a variety of snake venoms (Stringer *et al.*, 1971; Chang, 1979; White, 1981; Brook *et al.*, 1987; Ownby and

Colberg, 1988), but the myotoxic action of elapid and hydrophiid venom has not been extensively examined.

The venoms of the viperids are much more complex than those of the elapids and hydrophiids (Mebs *et al.*, 1983), being myotoxic, as well as affecting the vascular system of the muscle. Both the crotaline and viperine venoms are known to produce local effects (Tu, 1977), with hemorrhage, swelling, pain and muscle necrosis occurring in the vicinity of the envenomation site (deVries and Condrea, 1971; Stringer *et al.*, 1971; Tu, 1977; Chang, 1979). This damage can extend into muscle, tendon and cartilage, but is considered to be mostly localized. Death results from circulatory collapse and related problems, according to Chang (1979).

Elapid and hydrophiid venoms produce severe systemic effects (Tu, 1977), showing muscle necrosis, but only minor hemorrhage, swelling and pain (if any) at the site of envenomation (Homma, 1971; Tu, 1977). This is more prevalent with Australian elapids than with other elapid species (Mebs and Samejma, 1980).

Snake venoms are known to affect muscle in a variety of ways, which includes twitching, contracture, depolarization, decrease in excitability and necrosis. At least four venom components are thought to be responsible for these responses, according to Chang (1979). They are cardiotoxins, crotamines, proteases and phospholipase A.

Cardiotoxins, membrane-active polypeptides known to cause necrosis in skeletal muscle, caused disorganizing activities of the muscle membrane resulting in permeability changes that allowed for leakage of potassium, sodium and calcium ions (Ownby *et al.*, 1976; Chang, 1979). This results in the depolarization and contraction of the muscle, with necrosis of the muscle following. Chang (1979) points out that cardiotoxins may or may not be present in Australian elapid venoms, with some indications of its effects on muscle suggested by White (1981), but of only minor importance. Chang (1979) felt that if cardiotoxin is present in hydrophiid venom it would be of little importance, as is the case in elapids. A myotoxic fraction separated from the venom of *Enhydrina schistosa* by Geh and Toh (1976) and Fohlman and Eaker (1977) proved only to have phospholipase A activity.

Mebs and Samejma (1980) and Brook *et al.* (1987) suggested that widespread necrosis observed in muscle fibre, as the result of sea snake envenomation, resulted

from a type of myotoxic phospholipase A. According to Karlsson (1979), some of the most potent venom toxins of snakes, are either phospholipase A or contain a subunit of phospholipase. There are great differences in toxicity among the various phospholipase A enzymes (Chang, 1979), with specific types of myonecrosis induced by each different type (Ownby and Colberg, 1988). Phospholipase A from sea snake venom is known to cause muscle membrane depolarization through specifically hydrolyzing membrane phospholipids, as reported by Ibrahim (1970). This subsequently affects muscle integrity and ability to function (Tu, 1977). Other myonecrotic toxins accompanied by phospholipase A (Harris *et al.*, 1975) are more easily spread throughout envenomated muscle, due to the enzymes present, with neither the toxins nor the enzyme being as toxic on their own. There is thus a synergism between the two.

Ownby and Colberg (1988) divided muscle necrosis into the following four phases, based on muscle ultrastructural changes of envenomated individuals over time.

1. **Early phase** - initial stage of envenomation with a wide variety of pathological changes observable.
2. **Intermediate phase** - changes in the initial pathological states.
3. **Late phase** - common pathological state observed throughout with the muscle appearing as a homogeneous amorphous mass.
4. **Final phase** - regeneration of muscle.

Variation occurs only in the first two phases, resulting from different venom doses and types, causing differences in muscle damage and phase duration. All ultrastructural variation is eliminated by the late phase, with only variation in phase duration possible.

Homma and Tu (1971) described two types of myonecrosis; myolytic necrosis as being free of hemorrhage and coagulative necrosis as being accompanied by hemorrhage. These were considered as only different states of pathological change in necrosis by Ownby and Colberg (1988) as they were dependent on sampling time.

Muscle regeneration following necrosis is largely dose dependent, with muscle age playing an important role. Mature muscle, according to Spence and Mason (1987),

is typically incapable of mitosis, but due to the presence of satellite cells scattered throughout the muscle (Mauro, 1961; Spence and Mason, 1987) regeneration can occur. Young muscle is more resilient in its response to envenomation, as it has more satellite cells present, greater mitotic abilities and is more resistant to venom than is mature muscle (Harris *et al.*, 1980). High venom doses, however, are thought responsible for satellite cell destruction, according to Queiroz *et al.* (1984), resulting in poor regeneration in all envenomated muscle. Harris and Maltin (1982) demonstrated necrosis in mammalian skeletal muscle as the result of envenomation by *Oxyuranus scutellatus* venom. Muscle repair was obvious, with the presence of immature muscle fibre within seven days and complete recovery within a month.

Muscle necrosis has been previously reported in humans, as the result of hydrophiid envenomation (Reid, 1956; Marsden and Reid, 1961; Reid, 1961; Barme, 1968; Halstead, 1970; Reid, 1975; Tu, 1977; Mebs, 1978; Chang, 1979; Reid, 1979; Mebs and Samejima, 1980; Minton and Minton, 1980). Symptoms for human envenomations are aches and stiffness of muscles in the arms, legs, neck and trunk (Halstead, 1970; Tu, 1977; Chang, 1979; Minton and Minton, 1980), and the presence of myoglobin in the urine (Reid, 1956; Tu, 1977; Mebs, 1978; Chang, 1979; Minton and Minton, 1980). Myoglobinuria, which is the result of muscle breakdown (Reid, 1956; Marsden and Reid, 1961; Reid, 1979) and indicative of extensive muscle damage, is associated with renal failure (Reid, 1956; Tu, 1977; Mebs, 1978; Sutherland, 1983). The protein myoglobin is released through muscle necrosis, picked up by the blood and filtered by the kidneys. While in the kidneys the myoglobin is thought to cause extensive damage to the filtration mechanism (Marsden and Reid, 1961; Barme, 1968; Tu, 1977; Sutherland, 1983). According to Reid (1979), all clinical and pathological evidence clearly indicates that hydrophiid venom is myotoxic to humans.

Tu (1977), who has also reported hydrophiid venom as being myotoxic to humans, indicates that it is neurotoxic to other organisms, with Limpus (1978) suggesting that muscle necrosis only occurs in higher vertebrates. Geh and Toh (1976) and Fohlman and Eaker (1977), contrary to Tu's (1977) findings, reported necrosis in mice as the result of a phospholipase A fraction isolated from *Enhydrina schistosa* venom. Zimmerman and Heatwole (1987) reported kidney damage in mice as the result of

envenomation with *Aipysurus laevis* venom.

Mebs and Samejma (1980) felt that a possible reason for the lack of evidence (and agreement) regarding myotoxic effects of hydrophiid venom is that test animals are killed too early, either by lethal doses of neurotoxins or by sacrifice before necrosis can occur. Detection is also difficult, according to Chang (1979) and Karlsson (1979), since its effects are much slower than those of neurotoxins. Reid (1979) mentioned necrosis in human patients that was not detected until long after hospital discharge.

In light of the known occurrence of muscle necrosis in humans and laboratory mice, the lack of evidence for it in lower vertebrates (owing to the lack of work and overshadowing by the more toxic neurotoxins) and the recognized affinities between hydrophiids and elapids, a study of potential necrotic effects of hydrophiid venom was warranted.

The main objective of this thesis was to examine the effects of venom of *Aipysurus laevis* on its prey species. Muscles responsible for ventilatory movements, a known target of hydrophiid venom, were examined to determine if myotoxic effects occurred. Ventilatory rates and behavioural changes, as the result of envenomation, were also examined. These investigations were carried out using both whole venom and venom fractions.

In past studies with venom, investigators working with laboratory animals or referring to clinical aspects of human envenomation, did not treat venom in its ecological context as a feeding adaptation. More recent examples such as (1) Minton and Minton (1981) reporting lethal toxicity of five Australian elapids against anuran and lizard species and (2) Mackessy (1988) describing changes in the venom of *Crotalus viridis helleri* (Pacific rattlesnake) as related to diet, approach this problem. Another objective of this thesis was to examine the ecological significance of the venom of *Aipysurus laevis*.

1.2 Skeletal Muscle

1.2.1 General information

Skeletal muscle (striated muscle) represents one of the three major muscle types (Hainsworth, 1981; Stein, 1981), and is one of the concerns of this study, due to the suspected action upon it by hydrophiid venom.

Striated muscle cell morphology is different from that of other cells, according to DeRobertis *et al.* (1970) and Bloom and Faucett (1975); cells are elongated with several nuclei. A more important difference is that the bulk of the muscle is occupied by a contractile system (Smith, 1964; Porter and Bonneville, 1973) serving the cell's primary function.

1.2.2 Muscle organization

As seen in figure 1.1, muscle is made up of many individual fibres, with the cellular level of muscle collectively referred to as a fasciculus or fascicle (Bloom and Faucett, 1975; Hainsworth, 1981; Stein, 1981; Spence and Mason, 1987). Within the muscle fibre, extending the length of the muscle cell, are the myofibrils (Huxley, 1965; Harrington, 1981), containing actin and myosin filaments (DeRobertis *et al.*, 1970; Bloom and Faucett, 1975; Tu, 1977; Spence and Mason, 1987). It is at the level of the myofilaments (Porter and Bonneville, 1973; Hainsworth, 1981; Harrington, 1981) that the fundamental unit of muscle contraction, the sarcomere, is found. The striated appearance of skeletal muscle is the result of the regular organization of the myofilaments , with each myofibril exhibiting repeating bands, generally aligned in phase with successive fibrils across the muscle fibre (Bloom and Faucett, 1975). According to DeRobertis *et al.* (1970), the repeating bands of the myofibrils define the boundaries of the sarcomere, with band widths changing as the result of muscle contraction and relaxation.

On the peripheral ends of the sarcomere are the Z lines (Fig. 1.1D), which mark the limits of the sarcomere (Porter and Bonneville, 1973) and where the actin myofilaments are anchored (Harrington, 1981; Spence and Mason, 1987). Proceeding

inwardly from the Z lines are the lighter I bands, resulting from the thin actin myofilaments, in contrast to the darker A band located in the center of the sarcomere. The H zone, located in the center of A band, is much lighter in appearance than the rest of the band. This is due to the presence of only myosin myofilaments within the zone, with an overlap of myosin and actin myofilaments in the remainder of the A band giving it its darker appearance. The most centrally located M line is the result of a protein complex, which serves as a connecting bridge for the myosin myofilaments in each half of the sarcomere (DeRobertis *et al.*, 1970; Porter and Bonneville, 1973; Bloom and Faucett, 1975; Hainsworth, 1981; Harrington, 1981; Spence and Mason, 1987). It is at the level of the A band, according to Smith (1964) and DeRobertis *et al.* (1970), that the contraction and relaxation of the muscle occurs through the sliding of the actin and myosin myofilaments over each other.

1.2.3 Muscle ultrastructure

The sarcolemma (plasma membrane), which surrounds the muscle fibre, is a polarized membrane with an electrical potential (Bloom and Faucett, 1975; Stein, 1981). The myofibrils are surrounded by the sarcoplasm, which is the matrix or ground substance in which the nuclei, mitochondria, sarcoplasmic reticulum and various granular inclusions, i.e. glycogen, are found (Smith, 1964; DeRobertis *et al.*, 1970; Hainsworth, 1981). Other organelles are also present, such as golgi apparatus, but are not visible in routine preparations (Bloom and Faucett, 1975).

The muscle cell is typically multinucleated, with elongated nuclei generally found on the periphery of the fibre (Spence and Mason, 1987). The number of the nuclei present depend on the muscle length, type and the animal species (Bloom and Faucett, 1975).

The mitochondrial content in striated muscle is variable, with more active muscle, according to Smith (1964), possessing a greater number. They are located on the cell periphery as well as between myofibrils, and their synthesis of ATP, through oxidative phosphorylation and the Krebs cycle, is important for muscle contraction (Smith, 1964; Bloom and Faucett, 1975).

The sarcoplasmic reticulum, according to Spence and Mason (1987), is a vesicular

system comparable to the endoplasmic reticulum of other cells. Its responsibility is for the transmission of excitation transversely in the muscle fibre (Franzini-Armstrong and Porter, 1964), keeping everything in phase during muscle contraction (Smith, 1964). It extends longitudinally along and around the surface of each myofibril (DeRobertis *et al.*, 1970; Bloom and Faucett, 1975; Hainsworth, 1981) at the position of the A band in tubular form, referred to as sarcotubules or longitudinal tubules (Harrington, 1981; Stein, 1981). Two terminal cisternae of the sarcoplasmic reticulum are found at the level of the I band, with a transverse tubule continuous with the sarcolemma, sandwiched between them. Cumulatively these structures are termed the triad (Franzini-Armstrong and Porter, 1964; Smith, 1964; Porter and Bonneville, 1973; Bloom and Faucett, 1975; Stein, 1981); it is responsible for the conduction of the stimulus from the sarcolemma to the muscle fibres, resulting in a coordinated contraction and subsequent relaxation throughout the muscle (DeRobertis *et al.*, 1970; Hainsworth, 1981).

1.2.4 Muscle contraction

Motor neurons supply the muscle with the stimulus required for contraction (Spence and Mason, 1987) by way of the synapse of the neuromuscular junction (Florey, 1966), as discussed in section 1.1.3. The combining of the ACh with the appropriate receptor in the postsynaptic membrane initiates a permeability change in the membrane (sarcolemma). This change in permeability allows for the migration of ions across the membrane, particularly sodium and potassium, resulting in a change in membrane potential (depolarization), and produces an action potential that travels along the sarcolemma (Hoyle, 1957; Florey, 1966; DeRobertis *et al.*, 1970; Drachman, 1971; Russell and Brodie, 1974; Harder, 1975; Spence and Mason, 1987). This action potential passes along the transverse tubules into the center of the muscle triggering the release of calcium ions from the storage vesicles on the membrane of the terminal cisternae (Harrington, 1981). These ions bind to the tropin molecules of the thin filaments, allowing for myosin to combine with the actin portion of the filament, resulting in muscle movement. Active transport is responsible for pumping the calcium ions back to the cisternae thereby reversing the process (Huxley, 1965; DeRobertis

et al., 1970; Bloom and Faucett, 1975; Hainsworth, 1981; Harrington, 1981; Stein, 1981; Spence and Mason, 1987).

One of the intentions of this study is to examine changes in muscle at the ultrastructural level, focusing on the fibrils and organelles of fish envenomated with hydrophiid venom.

1.3 Fish Ventilation Mechanism

Unlike the internal lung with bidirectional movement of air, as found in terrestrial vertebrates, fish have an external gill and unidirectional flow of water over their ventilatory apparatus (Moyle and Cech, 1982). Water flow in most bony fish is accomplished by a branchial pump, which, according to Shelton (1970), represents a reduction in work as compared to a bidirectional system.

A continuous movement of water over the gills is accomplished by a synchronous expansion and contraction of the buccal and opercular chambers (Shelton, 1970; Wood and Lenfant, 1979). Movement of the two chambers is nearly simultaneous with the opening of the mouth (Hughes and Shelton, 1958) and only slightly precedes that of the opercular shields. The synochronization of the pumps, which is said by Moyle and Cech (1982) to accelerate water over the gills, maintains a positive differential pressure gradient across the gills and allows for gas exchange to take place between the water and circulatory system (Wood and Lenfant, 1979). Because of the concurrence of the pump activities, the presence of buccal and opercular valves and an elaborate neuromuscular apparatus to coordinate pump and valve activities, the unidirectional flow of water for ventilation is maintained (Hughes and Shelton, 1958; Moyle and Cech, 1982; Hughes, 1984).

The ventilation process is initiated by the mouth opening and buccal chamber expanding, mainly in a ventral direction (Hughes, 1974). A negative pressure is created in the chamber, with respect to the surrounding water (Hughes and Shelton, 1958; Lagler *et al.*, 1962), and water is channeled in through the mouth (Jones, 1972). A positive pressure, however, is created in the buccal chamber, with respect to the opercular chamber (Hughes and Shelton, 1958; Wood and Lenfant, 1979;

Randall *et al.*, 1981; Moyle and Cech, 1982) initiating a flow of water into the two opercular chambers (Ballintijn and Hughes, 1965; Hughes, 1984). With expansion of the opercular cavity, complete closure of the mouth (Hughes, 1984) and subsequent contraction of the buccal cavity there is an increase in water flow into the opercular chambers (Hughes and Shelton, 1958). The expulsion of water by contraction of the opercular chambers and opening of the opercular shields allows for the ventilation cycle to be initiated again.

Ventilatory rates increase and compensate for hypoxic conditions (Moyle and Cech, 1982). Compensation is made by either or both of the following changes:

Ventilatory frequency : an increase in the number of buccal and opercular strokes / minute.

Ventilatory stroke volume : an increase in the volume of water being pumped / ventilatory stroke.

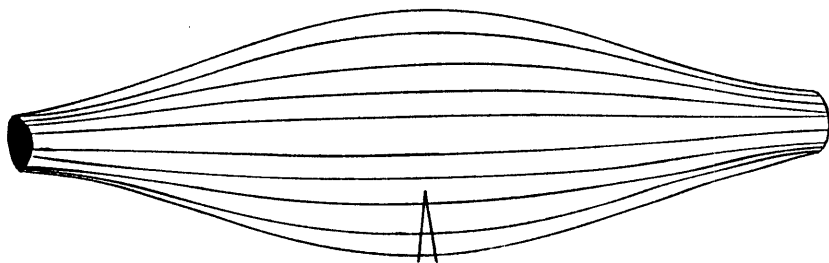
During these changes, as pointed out by Ballintijn and Hughes (1965), more of the head muscles of the fish become active, frequency of muscle activity increases and an exaggeration of the movements of the ventilatory parts is observed.

The workings of the muscles responsible for operating the component parts of the ventilatory mechanism are extremely complex, which according to Chaisson (1970), is because of the numerous moveable bones in the skulls of fish. Skeletal muscle, which makes up the bulk of fish muscle (Lagler *et al.*, 1962), is the muscle type servicing the ventilatory mechanism, and is considered by Harder (1975) to be the most suitable type; it is capable of short powerful contractions followed by a recovery phase.

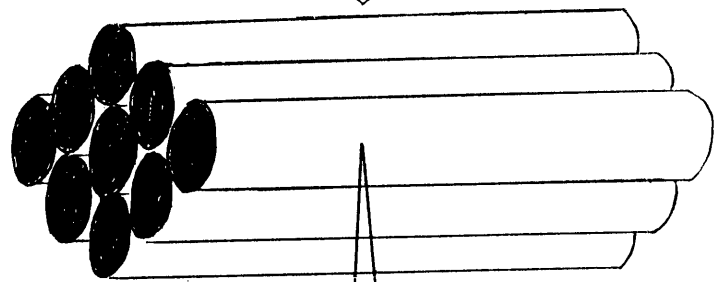
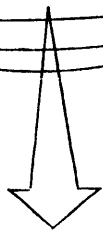
The expansion and contraction of the buccal and opercular chambers is through the action of various skeletal muscles, with the main one, as determined by electromyography (Ballintijn and Hughes, 1965) being the sternohyoideus muscle (Harder, 1975). According to Shelton (1970), there are interactions between skeletal muscles and tendons so that movement of one component causes complementary movement in many others. The contraction of the sternohyoideus muscle causes the hyoid arch to retract, which expands the branchial arch abducting the hyomandibula (Ballintijn

and Hughes, 1965; Shelton, 1970). Shelton (1970) pointed out that because of the hyomandibula's many important articulations it forms an important link between the skeletal and muscular components of the ventilatory mechanism. Through its abduction there is an expansion of both the buccal and opercular cavities, as well as an opening and widening of the mouth. The contraction of the chambers is brought about by the reversal of these events, and is accompanied by relaxation of the sternohyoideus muscle.

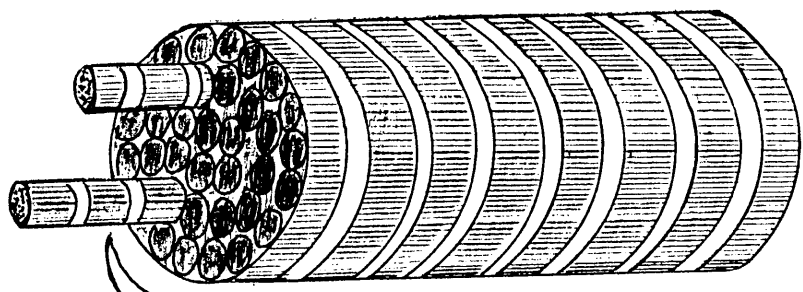
All ventilatory movements are coordinated by the medulla oblongata, which in fish (Hughes and Shelton, 1958) appears to be a relay station between the brain and spinal cord. Spinal nerves innervate the muscles of the ventilatory mechanism, as is the case of all somatic musculature of fish except for eye muscles, and originate from the anterior portion of the spinal cord (Harder, 1975). It is at the level of the nerve and muscle, as suggested in section 1.1, that venoms such as that of *Aipysurus laevis* are thought to affect the ventilatory mechanism.



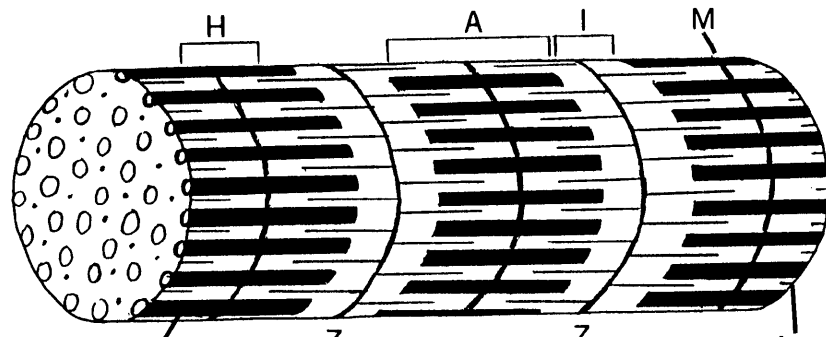
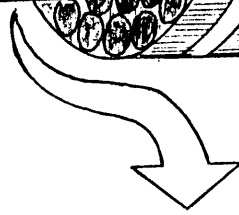
A



B



C



D

Chapter 2

General Materials and Methods

2.1 Venom Collecting and Processing

Aipysurus laevis (Fig. 2.2) venom was obtained on research cruises to the Swain Reefs (Fig. 2.1) in January 1985 and January 1986 aboard the T.S.M.V. *Australiana*, out of Gladstone, Queensland.

A. laevis was hand-collected by scooping up in mesh collecting bags while diving (Fig. 2.3) and transferred in plastic garbage bins by dinghy back to the ship for milking.

The snakes were milked by placing a plastic capillary tube held in forceps, over each fang and applying gentle pressure (Fig. 2.5). Two to three successive milkings were carried out on each fang (Fig. 2.4) in order to obtain maximum yields. The capillary tubing was then sealed with Adams Seal-Ease and placed into the ship's deep freeze.

The frozen venom was transported to the University of New England where it was defrosted at room temperature, removed from the capillary tubing by forcing air through the tubing with a small syringe, and placed into a collecting vial. It was then refrozen and freeze dried in an Edwards high vacuum freeze drier until no further weight change occurred.

This procedure took approximately seven days. The powdered venom was stored in a parafilm sealed vial in a deep freeze until required.

2.2 Venom Injection Procedures

Injection of experimental subjects with physiological saline (the carrier substance for the venom) and different dosages of *Aipysurus laevis* venom was carried out in accordance to table 2.1. This table shows the necessary adjustments in injection volumes for each dose for a ten gram fish, the stock solution used and the method of calculating the proper dose for subjects of different weights. Venom dosages are expressed in mg venom / kg body weight of the experimental subject.

The fish were weighed in a beaker containing saltwater on a Sartorius 1203 M.P. top loading balance to the nearest tenth of a gram. After weighing, lengths from the snout to the caudal fork were measured with calipers.

Injection was carried out using a 0.45 x 13 mm needle and an Agla Micrometer Syringe with an error factor of $\pm 0.05 \mu$. The syringe was fastened permanently to a ringstand, which made for easier handling.

Injections were given intramuscularly in the epaxial muscle of the caudal peduncle, as shown in figure 2.11. Care was taken not to cause unnecessary internal or external damage to the subject. The needle was removed from the injection site slowly with a slight inward and outward movement before complete removal. This prevented venom from leaking out of the injection site.

Handling of the fish was carried out quickly, but with great care, in order to minimize stress. Immediately after injection, each fish was transferred to the experimental holding facilities described in section 2.5.

2.3 Prey Species and Collecting Procedures

The studies with marine fish were carried out at the Heron Island Research Station (Fig. 2.1). From among the fish known to be preyed upon by *A. laevis* on the basis of previous stomach analyses (McCosker, 1975; Voris and Voris, 1983; Burns, 1984), species were chosen that were readily available and could be easily captured and maintained in captivity. They were:

1. *Chromis nitida*: Shining puller (Fig. 2.6)

2. *Chromis atripectoralis*: Blue puller (Fig. 2.7)
3. *Dascyllus aruanus*: Humbug (Fig. 2.8)
4. *Istiblennius meleagris*: Peacock blenny (Fig. 2.9)
5. *Istiblennius edentulus*: Rippled blenny (Fig. 2.10)

Permission to collect the fish was granted by the Great Barrier Reef Marine Parks Authority and the Queensland National Parks and Wildlife Service permit numbers G606, G86/093, G86/261, G88/061, 1910, 2092, and N88118. Fish traps were utilized initially, but resulted in low efficiency and little ability to select particular species.

For collecting *C. nitida* and *C. atripectoralis* the chemical anesthetizer quinaldine was used. According to Gibson (1967), it creates no problems for fish when administered in low doses. To 45 ml of concentrated quinaldine 55 ml of 100% ethanol was added, which was further diluted in approximately 400 ml of saltwater. The anesthetizer was sparsely applied to small coral heads containing *Chromis* species; once it took affect the anesthetized fish were carefully netted and placed into plastic holding-bags, with minimal handling; the water was changed every few minutes. The fish were brought to the boat at an ascent rate of approximately 2 m / min to prevent swim bladder injury and were then transferred to a floating flow-through holding bin. Transport to the holding facility, described in section 2.4, was within 15 minutes of departure from the collecting site. The holding bin was heavily aerated and protected from temperature elevation during transport.

Capture of *D. aruanus* was less difficult and more efficient than was true for the two *Chromis* species. The fish were corralled into a small coral head, which was placed into a net and brought to the surface at the ascent rate previously mentioned. At the surface the fish were carefully removed from the coral and placed into the floating flow-through holding bin. Transport to the holding facility was the same as that described for the *Chromis* species.

I. meleagris and *I. edentulus* were easily collected under rocks at night during low tide. Care was taken in handling during capture, but the mesmerizing effect of the light made their collection quite easy. The fish were held in a holding bin and

transferred to the holding facility within 10 minutes of departure from the collecting site.

All species underwent a 12 hour period where holding facility water was mixed with holding bin water to allow for temperature adjustments and fish acclimation.

Table 2.2 gives further information on collecting procedures, the prey species' habitats and the experiments to which they were subjected.

All surviving subjects were released at their approximate area of capture.

2.4 Stock Tank Holding Facilities

Stock tanks for holding experimental fish were maintained in the aquaria room of the Heron Island Research Station.

The fish were kept in 28 cm x 28 cm x 50 cm all-glass aquaria with P.V.C. piping providing shelter, and a screened top to prevent escape. A continual flow of seawater, filtered through a gravity-sand filter (Fig. 2.12), was delivered to each tank at a rate of 2 l / min. This flow provided adequate aeration as well as preventing changes in salinity, pH, temperature and other important water characteristics (King and Spotte, 1974).

No more than fifteen fish were held in an aquarium at one time, and all were fed Nutra Fin Staple Food for Tropical Fish once daily.

Daily maintenance of the system included checking drainage and water flow and the disposal of uneaten food and dead fish. Aquaria and contents were cleaned every three days and soaked for 12 hours in fresh water.

2.5 Experimental Tank Holding Facilities

Three experimental aquaria, 84.0 cm x 12.0 cm x 13.5 cm, were constructed from perspex, each containing seven compartments separated by grated dividers, which allowed for complete circulation of water (Fig. 2.13). In each aquarium six compartments, 12 cm x 10 x cm, contained one fish per compartment, with the seventh being used for an air-driven floss filtration system. Each experimental compartment had its

own aeration supply, controlled independently of other compartments. The aquaria were completely sealed, allowing for maintenance of water quality (King and Spotte, 1974), as well as preventing fish from escaping. During experiments, the fish were fed Nutra Fin Staple Food for Tropical Fish once daily.

Sea water used for the experiment was filtered for at least twelve hours, with a filter of diatomaceous earth, before adding it to the experimental system.

Beehive dividers were placed behind each aquarium with smaller pieces at the front of each compartment, providing adequate cover yet allowing for proper viewing.

Reflected light from a fluorescent tube behind the aquaria was used at night in order to keep lighting subdued and the fish at normal nocturnal rates of activity and ventilation. The aquaria were covered with dark plastic when night observations were not taking place in order to prevent interference from other light sources.

Maintenance included checking air flow, filtration rate and water level for each aquarium. Uneaten food and dead fish were immediately removed.

The aquaria and all components were cleaned, soaked in fresh water for 12 hours and filters changed after each experimental run.

2.6 Whitespot Prevention

Whitespot, which is an external infection from the ciliated hymenostome protozoan *Cryptocaryon irritans*, is responsible, according to Brown (1951) and Rohde (1982), for numerous fatalities in salt water aquaria. Stress makes fish more susceptible to the disease.

Hoffman and Meyer (1974) suggested that it can be treated and controlled along with the curing of the infected host with copper-based treatments such as copper sulfate and malachite green, but treatment proved difficult in the flow-through holding facilities. In the present study it proved to be a major problem in early experiments causing respiratory failure and death. The data from these experiments had to be discarded. Prophylactic procedures were therefore instituted.

Techniques discussed in sections 2.2 and 2.3 reduced stress during injections and collecting respectively, with sections 2.4 and 2.5 covering stress reduction during the

holding periods.

C. irritans is known to undergo a resistant cyst stage, making it necessary for the cleaning of each holding aquarium (Section 2.4) and experimental aquarium (Section 2.5) and contents. This was necessary to prevent subject contamination.

Other procedures employed to reduce whitespot were:

1. High amount of aeration in all holding systems.
2. Minimal useage of light during night observations.
3. Aquaria covered when night observations not taking place.
4. Access to holding facilities by investigator only.
5. Prevention of overcrowding in all holding facilities.
6. Constant monitoring of water quality in all holding facilities.

Once these procedures were placed into operation the whitespot disease was eliminated.

2.7 Electron Microscopy

The sternohyoideus muscle (Section 1.3) was removed from the experimental fish by microdissection. The fish were paralyzed by cutting their spines, and pinned out to expose the sternohyoideus muscle (Fig. 2.14), after which all skin was removed from the muscle area.

In order to minimize shrinkage of the muscle, as the result of primary fixation, it was necessary to preserve the origins and insertions (Davey, pers. comm.). This was accomplished by removing as an entity the lower mandible, ventral portion of the opercular shield and ventral pectoral girdle with the muscle (Fig. 2.14). The entire unit was immediately placed into the primary fixative (buffered glutaraldehyde) for fifteen minutes at 4°C.

The muscle tissue was cut into 2 mm pieces and processed through primary fixation, secondary fixation and ethanol dehydration as described by Hayat (1981).

A firm mix of Spurr's low-viscosity embedding resin (Spurr, 1969) was used for infiltration and embedding (Hayat, 1981), and BEEM capsules containing resin and sternohyoideus muscle tissue were polymerized at 58°C for twelve hours.

Sections of 60 nm to 100 nm thickness were obtained on a Porter-Blum MT-2 ultramicrotome and were stained with uranyl acetate and lead citrate according to procedures described by Lewis and Knight (1977).

The sections were examined and micrographs obtained using a Philips EM 300 Transmission Electron Microscope at the University of New England.

Figure 2.1: Map of the southern section of the Great Barrier Reef showing the Swain Reefs collecting site of *Aipysurus laevis*, and Heron Island were the experimental work took place. (From maps from G.B.R.M.P.A.)

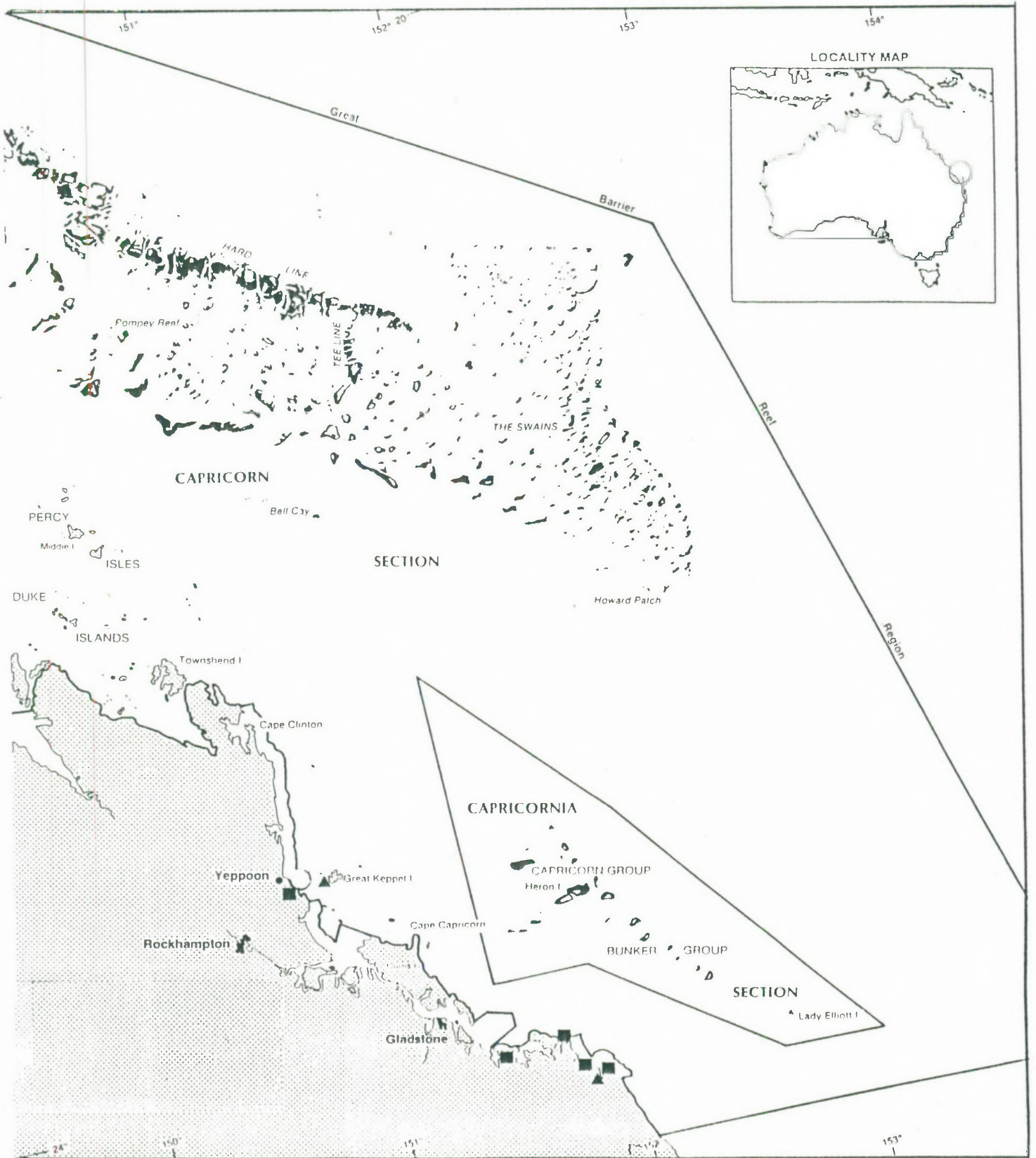


Figure 2.2: *Aipysurus laevis* (olive sea snake) in its natural habitat at the Swain Reefs of the Great Barrier Reef.

Figure 2.3: Collecting *Aipysurus laevis* by hand and carefully guiding it into a mesh collecting bag, which will be brought to the surface for experimental work.

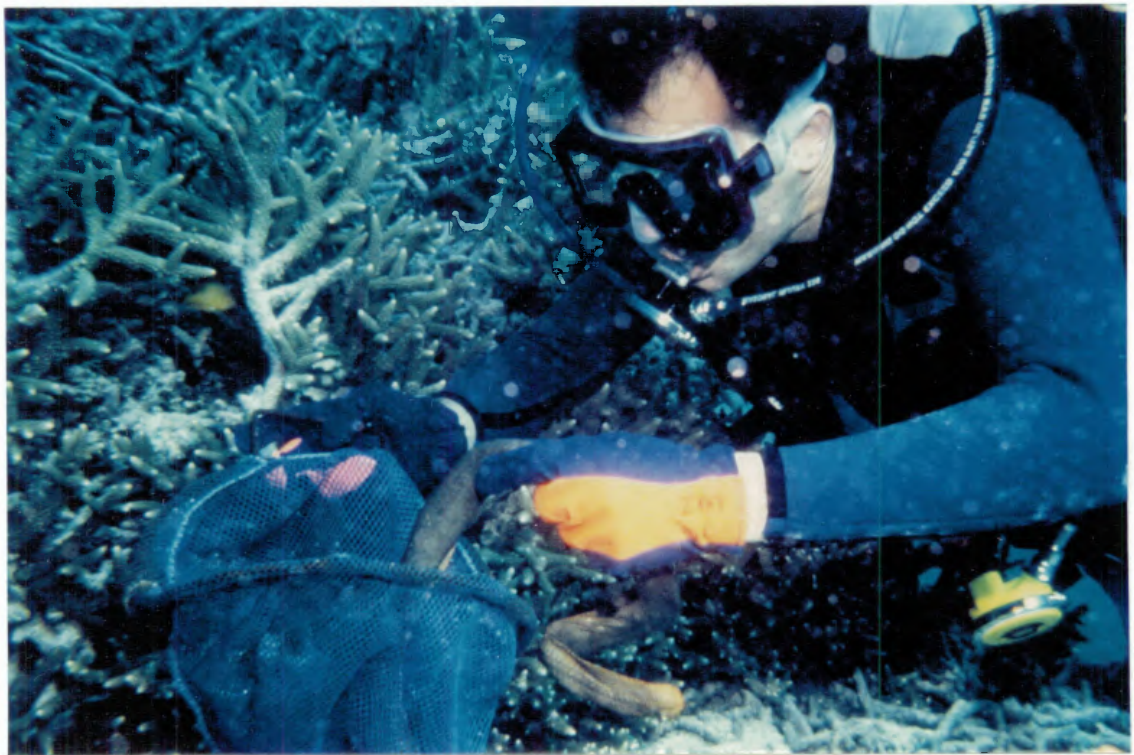


Figure 2.4: The fang of *Aipysurus laevis* with the skin sheath pulled aside exposing the fang.

Figure 2.5: Milking *Aipysurus laevis* with the use of plastic capillary tubing placed over each fang independently.



Table 2.1: *Aipysurus laevis* venom dosages used for the experimental procedures in mg venom / kg body weight of fish, with stock solution volume requirements and necessary adjustments in injection volumes for each dose calculated for a 10 g fish.

Actual volumes for each fish are determined by: fish weight (kg) / 10 x the calculated injection volume for the required dose.

Venom Dose (mg/kg)	Stock Solution (mg/ml)	Injection Volume (μ l)
4.000	1.000	0.040
2.500	1.000	0.025
0.750	0.250	0.030
0.500	0.250	0.020
0.300	0.125	0.024
0.270	0.125	0.022
0.250	0.125	0.020
0.200	0.125	0.016
0.180	0.125	0.014
0.150	0.125	0.012
0.100	0.125	0.008
0.085	0.125	0.007
0.075	0.125	0.006
0.060	0.063	0.010
0.050	0.063	0.008
0.010	0.016	0.006

Figure 2.6: *Chromis nitida* (Shining Puller)

Figure 2.7: *Chromis atripectoralis* (Blue Puller)



Figure 2.8: *Dascyllus aruanus* (Humbug)

Figure 2.9: *Istiblennius meleagris* (Peacock Blenny)

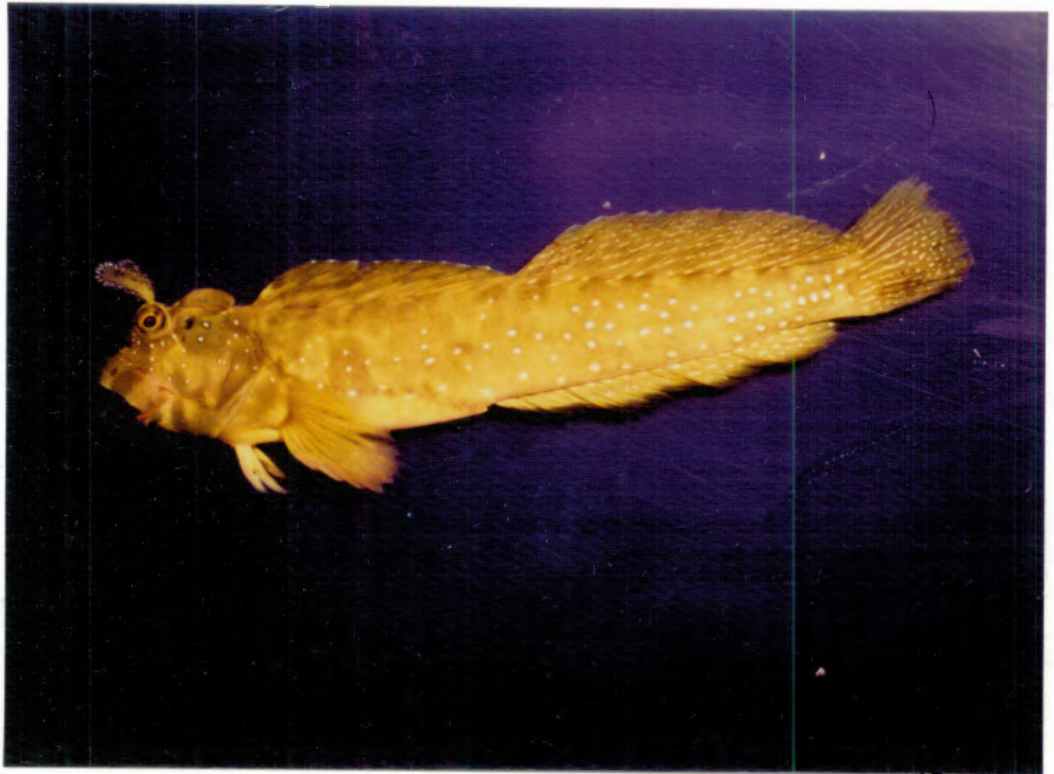


Figure 2.10: *Istiblennius edentulus* (Rippled Blenny)

Figure 2.11: Intramuscular injections were administered in the epaxial muscle of the caudal peduncle.



Table 2.2: The five prey species of marine fish used in the study, including habitat type, collecting techniques and experiments to which they were subjected.

Prey Species	General Information	Collecting Technique	Experimental Usage
<i>Chromis nitida</i> Shining Puller Fig 2.6	Very common on outer reef slope and adjacent shallows. Often occurring in large aggregations especially around branching corals. (Russell, 1983)	Anesthetizer Quinaldine	LD50 Study Ventilation Study Behavioural Study
<i>Chromis atripectoralis</i> Blue Puller Fig 2.7	Very common in protected shallow areas, reef crests and reef slopes. Found in large aggregations around branching corals. (Amesbury and Myers, 1982)	Anesthetizer Quinaldine	LD50 Study
<i>Dascyllus aruanus</i> Humbug Fig 2.8	Found on reef flats, and in coral outcrops adjacent to reef slopes. Common in small groups in small coral heads. (Russell, 1983; Amesbury and Myers, 1982)	Corralled into small coral heads, which are brought to the surface where fish are removed	LD50 Study Ventilation Study Behavioural Study Venom Fraction Study E.M. Study
<i>Istiblennius meleagris</i> Peacock Blenny Fig 2.9	Found under rocks, dead coral and in crevices in reef tidal flats. (Grant, 1982; Russell, 1983; Amesbury and Myers, 1982)	Capture by hand under rocks at night during low tide	LD50 Study Ventilation Study Behavioural Study
<i>Istiblennius edentulus</i> Rippled Blenny Fig 2.10	Found under rocks, dead coral and in crevices in reef tidal flats. (Grant, 1982; Russell, 1983; Amesbury and Myers, 1982)	Capture by hand under rocks at night during low tide	LD50 Study

Figure 2.12: Stock holding tanks showing the gravity- sand filter.

Figure 2.13: The experimental holding tanks showing the experimental compartments.

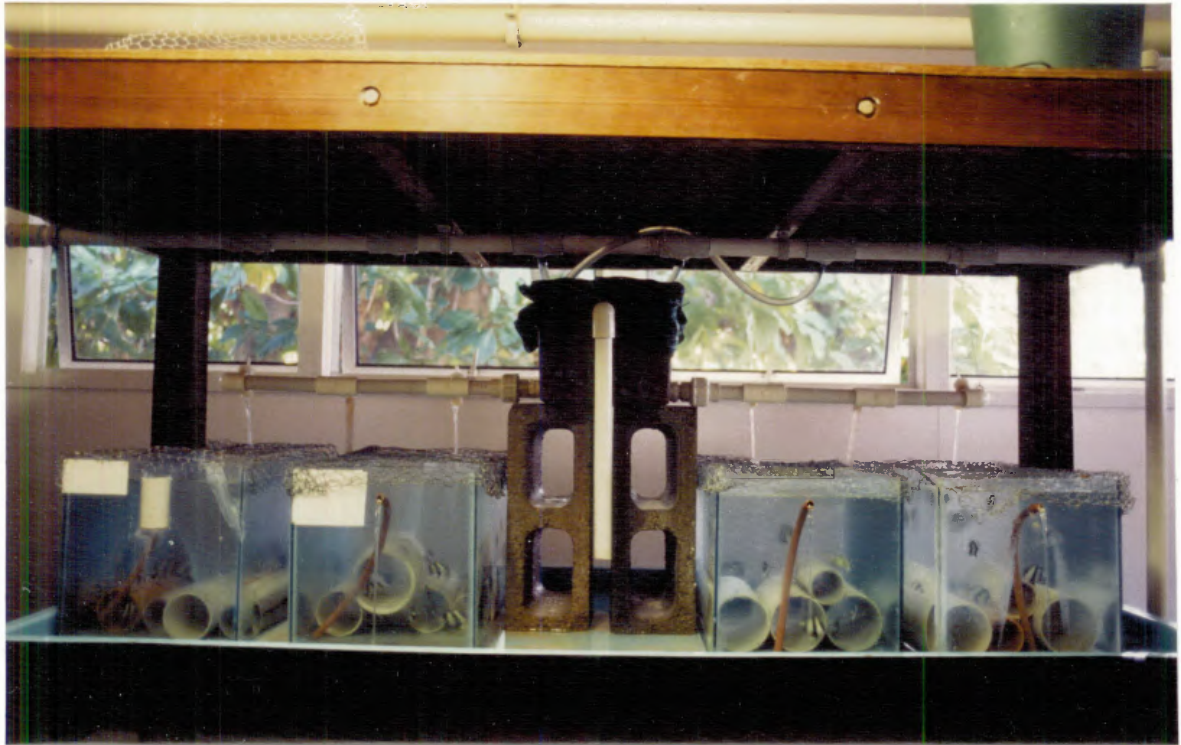
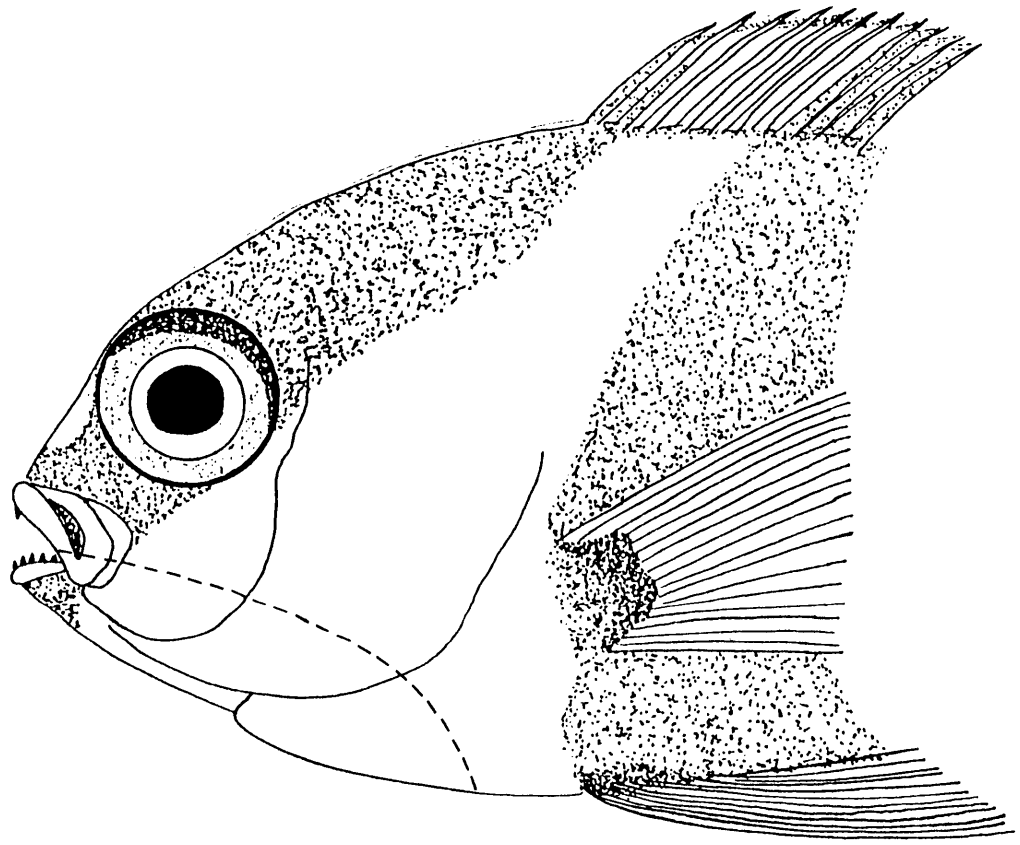


Figure 2.14: (Top) Lateral view of the head of *Dascyllus aruanus* and (Bottom) ventral view showing the areas cut to remove the sternohyoideus muscle as an entity preventing shrinkage of the muscle.



STERNOHYOIDEUS MUSCLE

