

Chapter 7

Ultrastructural Study

7.1 Introduction

Myotoxins have been previously found in a great variety of venoms (Chang, 1979; Ownby and Colberg, 1988), with muscle breakdown reported in humans as the result of sea snake envenomation (Reid, 1956; Marsden and Reid, 1961; Reid, 1961; Barne, 1968; Halstead, 1970; Reid, 1975; Mebs, 1978; Chang, 1979; Mebs and Samejima, 1980; Minton and Minton, 1980). No evidence, according to Limpus (1978), however, has been previously obtained for the occurrence of muscle necrosis in lower vertebrates, i.e. fish, as the result of this type of envenomation.

Based on the results of the ultrastructural examination of goldfish (*Carassius auratus*) muscle in chapter 3, a further study was warranted to examine muscle tissue of marine prey species envenomated with *Aipysurus laevis* venom.

The purpose of the study was to determine if the sternohyoideus muscle, the major muscle of the gill-ventilation system of fish (Section 1.3), was affected by the venom of *A. laevis* and if so, the the nature of the effects.

The study also tested a number of osmolarities of buffering solutions in order to select the one that affected muscle structural integrity least during processing for electron microscopy.

7.2 Materials and Methods

Dascyllus aruanus (Fig. 2.8) were captured and maintained according to procedures outlined in chapter 2, and were injected with 0.09 and 0.75 mg / kg *A. laevis* venom as described in section 2.2.

The sternohyoideus muscle was removed and processed for electron microscopy according to procedures outlined in section 2.7, except that filtered sea water replaced phosphate buffer during fixation. Sea water was necessary since marine organisms exist in an environment with a high concentration of salt as compared to freshwater and terrestrial animals. The latter have extracellular environments of 0.28 to 0.30 M, and the standard buffers for electron microscopy are set up for these molarities (Hayat, 1981); accordingly it makes them inappropriate for use with marine fish muscle.

In order to ascertain the correct osmolarity required to obtain close to normal muscle integrity (Section 1.2), a range of saltwater buffers were used to prepare material for electron microscopical study. The concentrations of saltwater used were 10, 24 and 32 parts / thousand, approximately 0.17, 0.41 and 0.55 M respectively. The saltwater was filtered through a 0.45 μ Milipore filter before use.

The salinity of the sea water selected for use in the main body of the study, based on average monthly readings, was 26 parts / thousand.

To further insure that muscle structure was maintained intact during handling, a study examining the effects of time following death on muscle deterioration was carried out. This was accomplished by removing the sternohyoideus muscle, as outlined in section 2.7, but with a five minute delay before the muscle was processed. Normally it took thirty seconds to remove the sternohyoideus muscle, so the additional four minutes and thirty seconds was adequate time to determine if significant muscle change independent of envenomation was occurring during tissue processing. Observations on heartbeat and body movements were carried out prior to and after muscle removal.

Transverse sections of control and envenomated sternohyoideus muscle were quantitatively analyzed and compared using VIDAS and IBAS image analysis systems,

employing a Hamamatsu C3077 camera, courtesy of Kontron Bildanalyse Eching, West Germany. Binary identification images were produced from original electron micrographs that were contrast- and contour-enhanced. From the binary images differences in swelling of the sarcoplasmic reticulum was determined by calculating area of coverage on the micrographs. Transverse sections were used, a least biased angle of examination for such studies, since the anisotropic pattern of muscle causes differences in patterns and shapes with different angles (Weibel, 1972).

7.3 Results

The osmolarity study verified suggestions by Johnson and Davey (pers. comm.), as well as findings by Hayat (1981), regarding the use of filtered sea water as a buffer for glutaraldehyde fixation. Davey (1973) suggested that the best vehicle for a fixing agent appears to be the physiological solution normally bathing the tissue, which in the case of marine fish is sea water.

Figure 7.3 demonstrates dilated sarcoplasmic reticulum as the result of a 10 parts / thousand sea water buffer, with less dilation seen in 24 parts / thousand (Fig. 7.2) and still less at 32 parts / thousand (Fig. 7.1).

Examination of the control (Figs. 7.4 and 7.5) and saline-injected control micrographs (Figs. 7.6 and 7.7) reveals only normal muscle morphology as described in section 1.2. The slight swelling of the sarcoplasmic reticulum is thought to result from the 26 parts / thousand sea water buffer, consistent with the osmolarity findings found throughout the controls. Figures 7.8 and 7.9 demonstrate muscle necrosis as the result of envenomation, with dilation of the triad, disappearance of the Z lines, difficulty of myofilament recognition and swelling of the sarcomere being apparent.

Dilation of the terminal cisternae of the sarcoplasmic reticulum, according to Stringer *et al.* (1972), is an initial indication of muscle degeneration, as observed in figures 7.10 and 7.11. With the onset of more advanced stages, the swelling increases (Figs. 7.12, 7.13 and 7.16), and it becomes increasingly difficult to distinguish the triads because of the random distribution of the terminal cisternae (Tu, 1977) and large vacuole-like structures replacing the triads (Figs. 7.14 and 7.15).

Shortly after the dilation of the terminal cisternae, the myofilaments become disaligned (Figs. 7.8 to 7.11) due to thickening of the myosin, and the actin myofilaments becoming less apparent (Stringer *et al.*, 1972). This is accompanied by a swelling of the fibers and sarcomeres (Harris *et al.*, 1975) referred to by Yokote (1982) as 'cloudy swelling'. According to Ghadially (1975), this myofibrillary degeneration (a term used to describe myofilament and Z line changes resulting from degenerative processes) causes a loss in normal muscle striation and banding patterns (Figs. 7.12 to 7.15).

The Z line, which maintains normal integrity during early necrosis, eventually acquires a zig-zag appearance (Fig. 7.19) referred to as Z line streaming (Ghadially, 1975). This condition may affect one or more sarcomeres in a myofibril or many adjacent myofibrils; sarcomeres become difficult to distinguish. During degeneration the Z line disappears along with other cell components.

With the progression of necrosis, the myofilaments coalesce into an indistinguishable amorphous mass (Figs. 7.12 to 7.15), and become less electron dense (Tu, 1977), giving an artifactual impression of a lower quality micrograph. The resulting homogeneous mass from the myofilament breakdown is referred to as hyaline degeneration (Yokote, 1982). Interfibrillar space increases with increasing degeneration, with the sarcoplasm possessing remnants of cellular material, also less electron dense (Fig. 7.17); this indicates a pathological change, rather than a mechanical one according to Yokote (1982).

During necrosis mitochondria undergo swelling, referred to as intramitochondrial edema, develop abnormal cristae, and eventually degenerate and lyse (Stringer *et al.*, 1972; Tu, 1977). Figure 7.18 shows swollen mitochondria as the result of envenomation, with abnormal cristae apparent. During later stages of necrosis (Figs. 7.14 and 7.15) mitochondria were not discernible and were assumed to have lysed. According to DiMauro *et al.* (1985), whenever mitochondria display structural changes, there is usually accompanying functional disorders.

Another sign of necrosis is an increase of glycogen granules scattered throughout the muscle (Tu, 1977). Figures 7.10 to 7.15 demonstrate a progressive increase in glycogen with increased time following envenomation.

Venom affects some fibers and the resultant intermixing of intact fibers with those undergoing necrosis gives muscle a mosaic appearance (Marsden and Reid, 1961; Harris *et al.*, 1975; Reid, 1975). The tissue sampled at 72 hours appeared to possess more normal fibers than tissue sampled at 96 and 120 hours, demonstrating progressive deterioration. The increased swelling of the sarcoplasmic reticulum, as well as its vacuolation, and the loss of an orderly structural pattern is also apparent in figures 7.10 to 7.15.

Differences between control and envenomated tissue are also observed in the transverse sections (Figs. 7.20 and 7.21). The sarcoplasmic reticulum and mitochondria are much more swollen in the envenomated tissue (Fig. 7.21) as compared to controls (Fig. 7.20), with mitochondrial cristae disrupted and difficult to distinguish.

Quantitative comparisons between control and envenomated tissue were obtained through image analysis of transverse sections of muscle tissue. Percentage area of the micrograph covered by sarcoplasmic reticulum was determined from the binary image (Figs. 7.23 and 7.25) obtained from image enhancement (Figs. 7.22 and 7.24).

The transverse section of the control had 12.9% of its area covered with sarcoplasmic reticulum. The transverse section of the tissue envenomated with 0.09 mg / kg of *Aipysurus laevis* venom and sampled 120 hours after envenomation had 31.2% of its area covered with sarcoplasmic reticulum. This more than doubling the area of the sarcoplasmic reticulum demonstrates a quantitative change in muscle morphology following envenomation.

Further analysis of the images could be carried out, i.e. examinations of changes of texture or shape and of proximities of structures to each other, but were not included in the present study.

The study of muscle deterioration during handling demonstrated that observed ultrastructural changes were the result of envenomation, and not of deterioration during tissue removal. Tissue left for five minutes before processing possessed the same morphology as the controls (Figs. 7.4 to 7.7), with no loss of integrity such as that observed in envenomated tissue. The muscle was removed while vital signs, i.e. heart beat and opercular movements, still persisted.

7.4 Discussion

Along with the preliminary evidence of chapter 3, this study demonstrates myotoxic effects of *Aipysurus laevis* venom on fish ventilatory muscles.

According to Mebs and Samejrma (1980), hyaline necrosis of muscle fiber is most probably due to myotoxic phospholipase A in sea snakes. Several of the most potent venom toxins are either phospholipase A or contain a subunit of phospholipase (Karlsson, 1979). Myotoxic phospholipase has been shown to affect the sarcoplasmic reticulum, but it is not known if the effects are direct or act indirectly through the release of cellular phospholipase (Gutierrez *et al.*, 1984).

Harris *et al.* (1980) stated that myotoxicity is not related to neural or mechanical activity of the muscle, and neurotoxins, according to Mebs (1978), do not produce muscle lesions.

As pointed out by Yokote (1982), any variation from normal muscle morphology, as seen in section 1.2, reflects change in the sarcoplasm and is a clear sign of a pathological condition. All signs seen in this study, such as dilation of the sarcoplasmic reticulum, myofibril degeneration, mitochondrial changes (swelling and change in the cristae) and Z line streaming, clearly indicate that *A. laevis* venom has myotoxic effects. These findings parallel those of Marsden and Reid (1961), Stringer *et al.* (1971), Stringer *et al.* (1972), Harris *et al.* (1975), Ghadially (1975), Reid (1975), Tu (1977), Mebs and Samejrma (1980) and Ownby and Colberg (1988), all demonstrating some degree of necrosis.

Varying states of muscle necrosis have been previously described by Ghadially (1975), who defines pathological changes in muscle as the result of degenerative causes. Ownby and Colberg (1988) divide necrosis into four phases (Section 1.1.4), with the early phase exhibiting a variety of changes in the muscle, but more homogeneous changes occurring during later phases. Only the first three phases of Ownby and Colberg's four were observed in the present study, with the signs of necrosis becoming more homogeneous with time. The regeneration phase, phase four, was not noted, probably because observations were terminated before it became evident.

A decrease in Ca-ATPase activity induced by myotoxins was observed by Gutierrez

et al. (1984) and Volpe *et al.* (1986). It resulted in an increase in calcium concentration inside the muscle cell, since no ATP was available for pumping it back into the sarcoplasmic reticulum. This increase in calcium ions, which are normally used in muscle contraction, causes hypercontraction of muscle fiber as well as mitochondrial poisoning and eventual muscle necrosis (Harris *et al.*, 1980; Harris and MacDonell, 1981).

Myotoxins are also known to cause a loss of muscle membrane integrity and a resulting increase in permeability. This change in permeability enhances the effects of other venom components, as well as causes a decrease in ion concentration, and thus a fall in resting membrane potential (Harris and MacDonell, 1981). Certain components found in crotalid venom affect the muscle plasma membrane (Ownby *et al.*, 1976) allowing for an influx of water into the sarcoplasmic reticulum. This surge of water into the sarcoplasmic reticulum causes dilation (an initial indication of necrosis in muscle) to occur (Stringer *et al.* 1972).

Mitochondrial morphological changes during necrosis may possibly be due to their own activity, according to Stringer *et al.* (1971). They become hyperactive after envenomation, with swelling and loss of organization of the cristae thought to be related to inhibition of respiration (Stringer *et al.*, 1971; DiMauro *et al.*, 1985). It was previously discovered that there is an uncoupling of the electron transport system as the result of elapid venom (Stringer *et al.*, 1971), with Harris *et al.* (1975) reporting tiger snake toxin affecting mitochondrial enzymes. Fohlman and Eaker (1977) and Tu (1977) also reported a myotoxic phospholipase from *Enhydrina schistosa* as specifically inhibiting mitochondrial respiration in muscle. This inhibition of respiration may also be responsible, according to Stringer *et al.* (1971), for glycogen accumulation. Normally it is metabolized by the mitochondria.

Previous studies (Minton, 1967; Rosenberg, 1967; Barne, 1968; deVries and Condrea, 1971; Porter, 1972; Minton, 1974; Tu, 1977; Chang, 1979; Minton and Minton, 1980) and reports on myoglobinuria and other muscle-related problems in envenomated humans (Reid, 1956; Marsden and Reid, 1961; Reid, 1961; Barne, 1968; Halstead, 1970; Reid, 1975; Mebs, 1978; Chang, 1979; Mebs and Samejima, 1980) converge to indicate that muscle necrosis occurs after envenomation by elapid

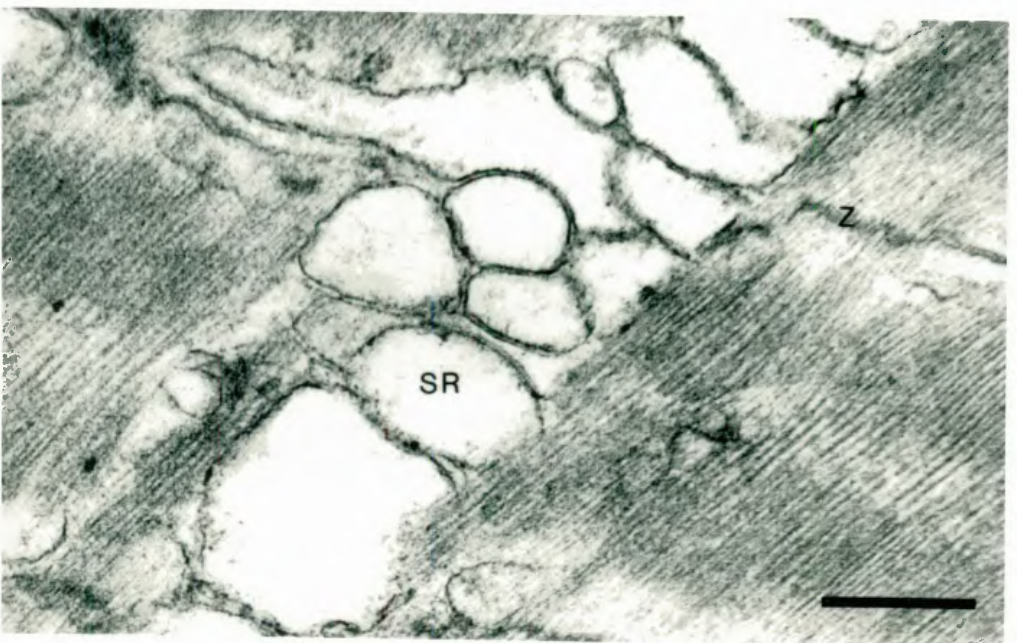
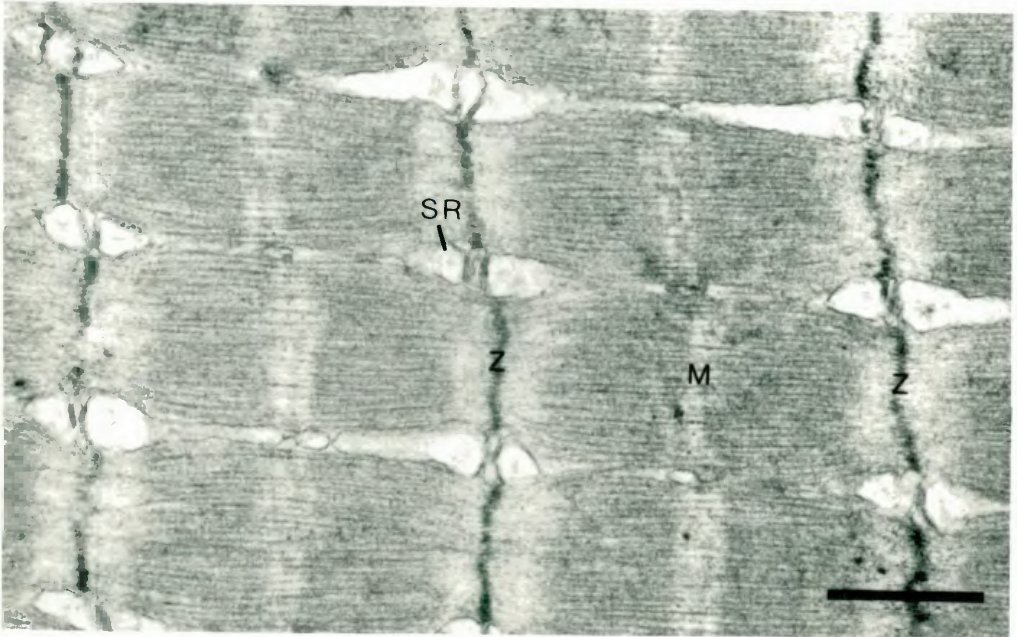
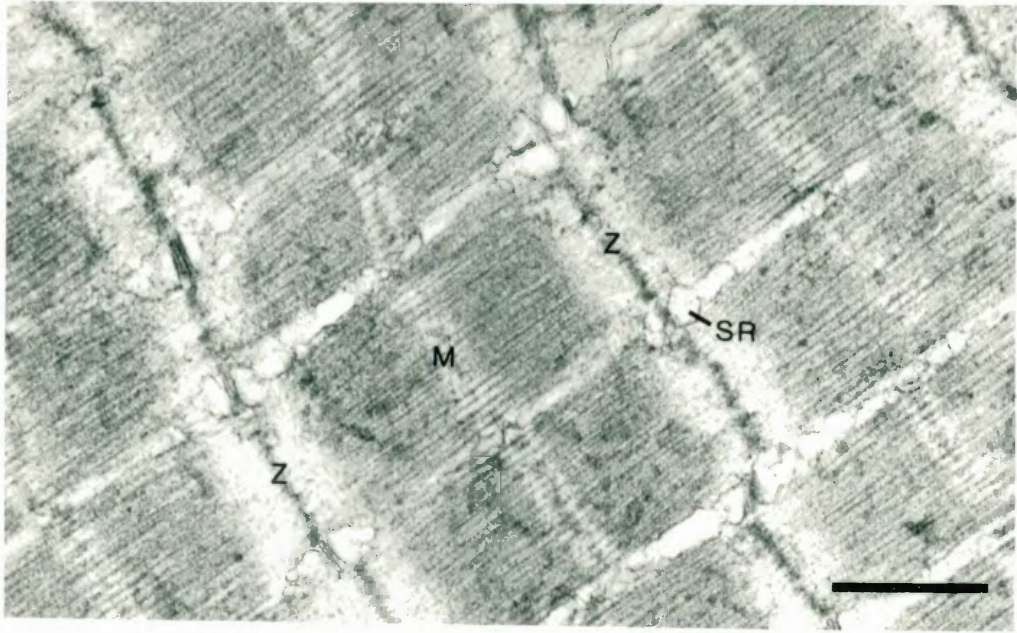
and hydrophiid snakes. The present study demonstrates that *Aipysurus laevis* venom contains myotoxic components affecting prey fish.

An explanation for the lack of previous evidence of necrosis in lower vertebrates (i.e. marine fish) as the result of sea snake venom, is that myotoxic effects are much slower acting than are the highly lethal neurotoxins (Chang, 1979; Karlsson, 1979). The latter may cause death before necrosis occurs. Even if low venom doses are used, premature sacrifice of experimental animals may prevent detection of necrosis.

Figure 7.1: Sternohyoideus muscle from *Dascyllus aruanus*, with 32 parts / thousand sea water replacing the phosphate buffer during fixation procedures for electron microscopy. Normal sarcoplasmic reticulum seen here, M - M line, SR - sarcoplasmic reticulum, Z - Z line (Bar = 0.51 μm).

Figure 7.2: Sternohyoideus muscle from *Dascyllus aruanus*, with 24 parts / thousand sea water replacing the phosphate buffer during fixation procedures for electron microscopy. Sarcoplasmic reticulum swelling seen here, M - M line, SR - sarcoplasmic reticulum, Z - Z line (Bar = 0.51 μm).

Figure 7.3: Sternohyoideus muscle from *Dascyllus aruanus*, with 10 parts / thousand sea water replacing the phosphate buffer during fixation procedures for electron microscopy. Extensive swelling of the sarcoplasmic reticulum seen here, M - M line, SR - sarcoplasmic reticulum, Z - Z line (Bar = 0.41 μm).



Figures 7.4 through 7.9 present sternohyoideus muscle from *Dascyllus aruanus*, SR - sarcoplasmic reticulum, Z - Z line.

Figure 7.4: Control group showing normal muscle morphology, with minimal swelling of the sarcoplasmic reticulum possibly due to the salt water buffers (Bar = 0.65 μm).

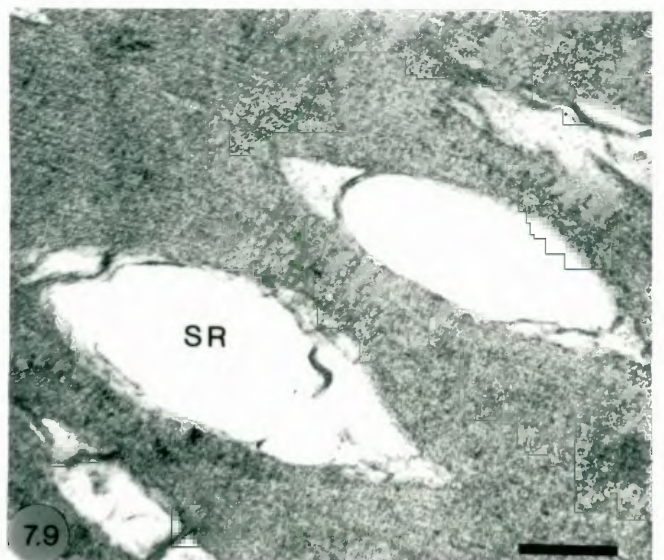
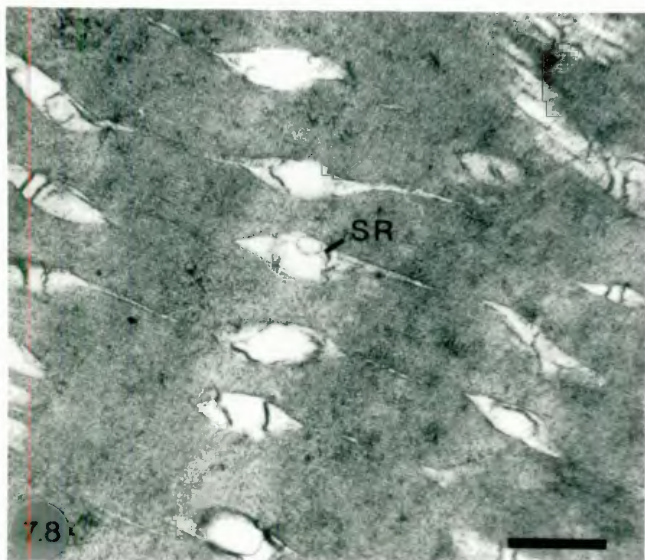
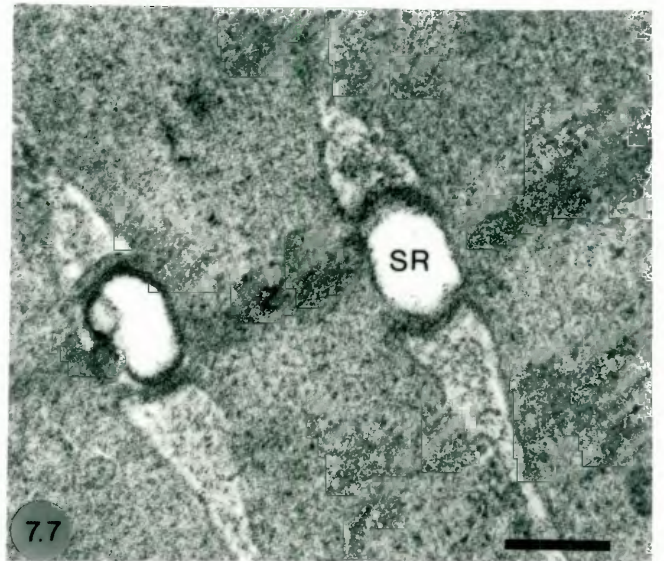
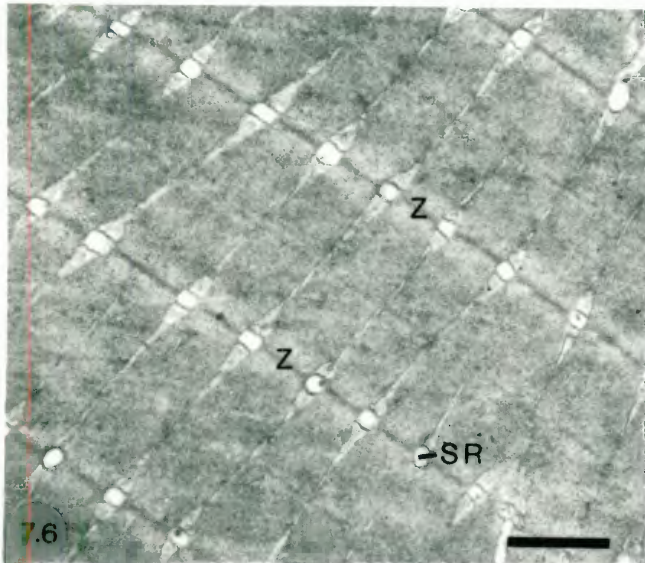
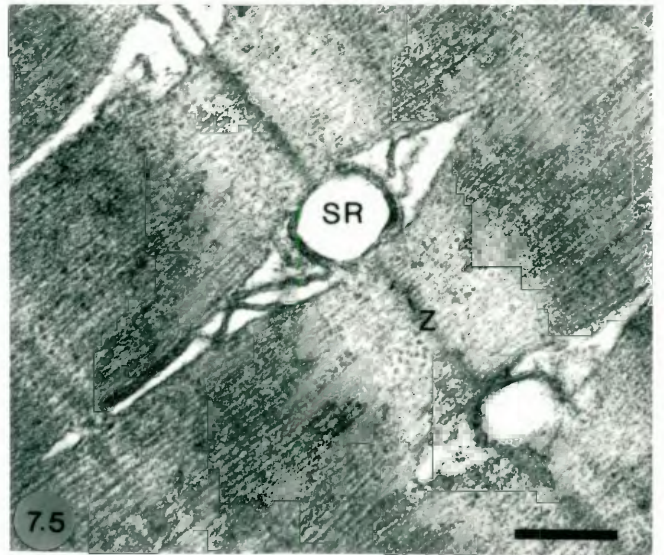
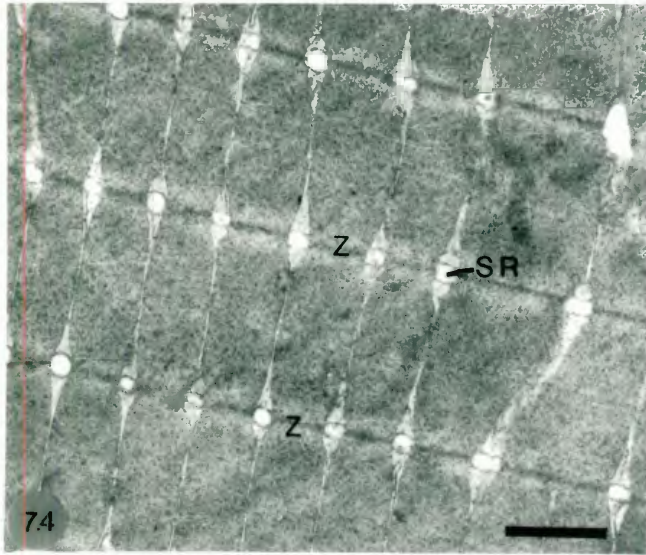
Figure 7.5: Higher magnification of control group showing normal muscle morphology (Bar = 0.19 μm).

Figure 7.6: Saline-injected control group showing normal muscle morphology, with minimal swelling of the sarcoplasmic reticulum possibly due to the salt water buffers (Bar = 0.65 μm).

Figure 7.7: Higher magnification of the saline-injected control group showing normal muscle morphology (Bar = 0.13 μm).

Figure 7.8: Group envenomated with 0.75 mg / kg *Aipysurus laevis* venom showing swelling of the sarcomere and sarcoplasmic reticulum, with Z lines and myofilaments difficult to distinguish (Bar = 0.53 μm).

Figure 7.9: Higher magnification of the envenomated group showing the swollen triad of the sarcoplasmic reticulum (Bar = 0.22 μm).



Figures 7.10 through 7.15 present sternohyoideus muscle from *Dascyllus aruanus* envenomated with 0.09 mg / kg *Aipysurus laevis* venom, G - glycogen granules, HD - hyaline degeneration, SR - sarcoplasmic reticulum, Z - Z line.

Figure 7.10: Muscle sampled at 72 hours after envenomation showing swelling of the sarcomere and sarcoplasmic reticulum (Bar = 0.84 μm).

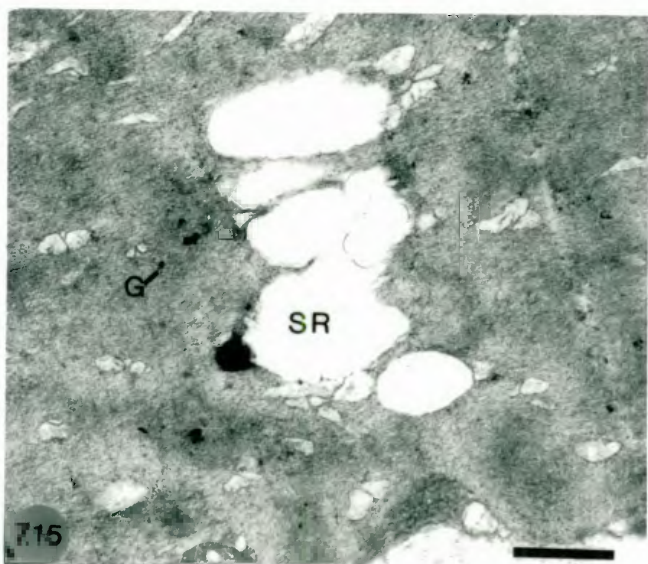
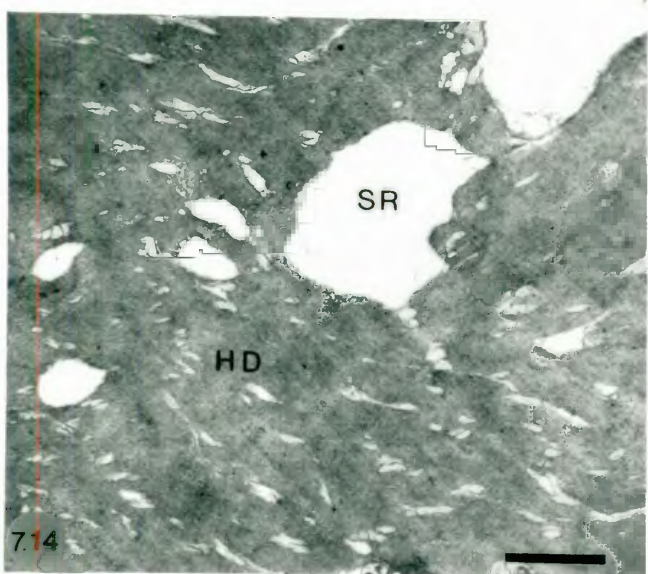
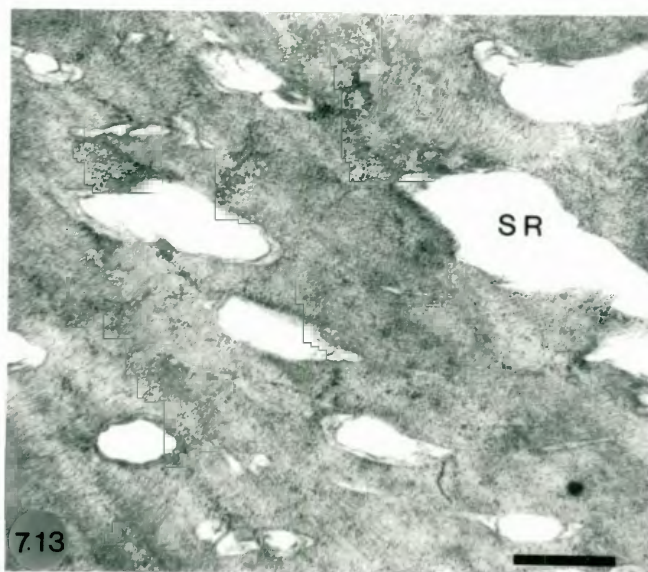
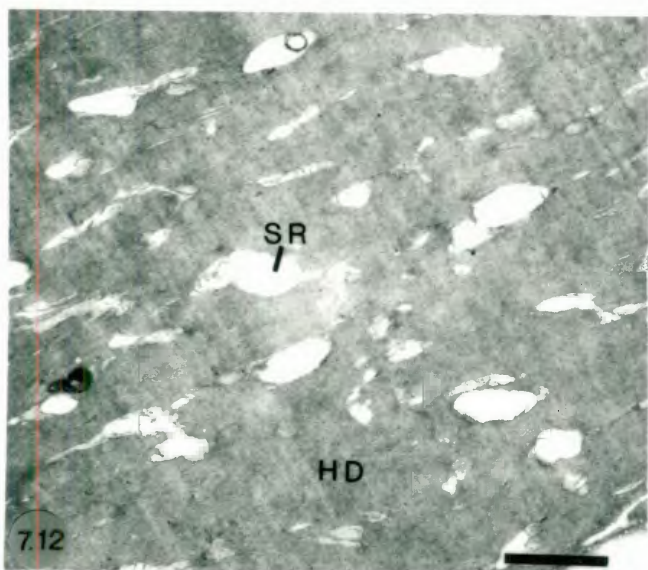
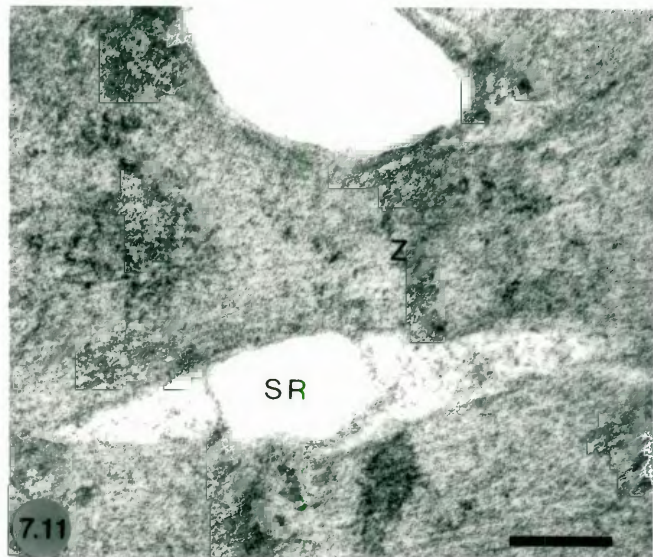
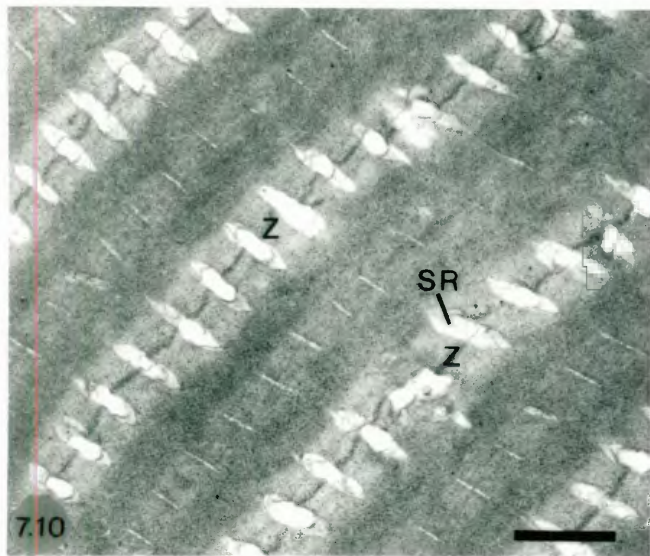
Figure 7.11: Higher magnification of muscle sampled at 72 hours after envenomation showing swelling of the sarcoplasmic reticulum (Bar = 0.13 μm).

Figure 7.12: Muscle sampled at 96 hours after envenomation showing increased swelling and organizational breakdown of the sarcoplasmic reticulum and hyaline degeneration (Bar = 1.08 μm).

Figure 7.13: Higher magnification of muscle sampled at 96 hours after envenomation showing increased swelling and organizational breakdown of the sarcoplasmic reticulum (Bar = 0.53 μm).

Figure 7.14: Muscle sampled at 120 hours after envenomation showing vacuolation of the sarcoplasmic reticulum and hyaline degeneration (Bar = 1.3 μm).

Figure 7.15: Higher magnification of muscle sampled at 120 hours after envenomation showing vacuolation of sarcoplasmic reticulum and high degree of disarray of muscle (Bar = 0.65 μm).



Figures 7.16 through 7.21 present sternohyoideus muscle from *Dascyllus aruanus* envenomated with *Aipysurus laevis* venom (except Fig. 7.20), C - cristae, CR - cellular remnants, HD - hyaline degeneration, IS - interfibrillar space, M - mitochondria, SR - sarcoplasmic reticulum, Z - Z line.

Figure 7.16: Muscle showing vacuolation of the sarcoplasmic reticulum and hyaline degeneration, with difficulty in distinguishing muscle banding (Bar = 0.39 μm).

Figure 7.17: Muscle showing separation of interfibrillar space with remnants of cellular material (Bar = 1.08 μm).

Figure 7.18: Muscle mitochondria showing intramitochondrial edema and abnormal cristae (Bar = 0.39 μm).

Figure 7.19: Muscle showing Z line streaming and swelling of the sarcoplasmic reticulum (Bar = 0.39 μm).

Figure 7.20: Transverse section of control muscle showing normal muscle morphology (Bar = 0.69 μm).

Figure 7.21: Transverse section of envenomated muscle showing swelling of sarcoplasmic reticulum and mitochondria (Bar = 0.69 μm).

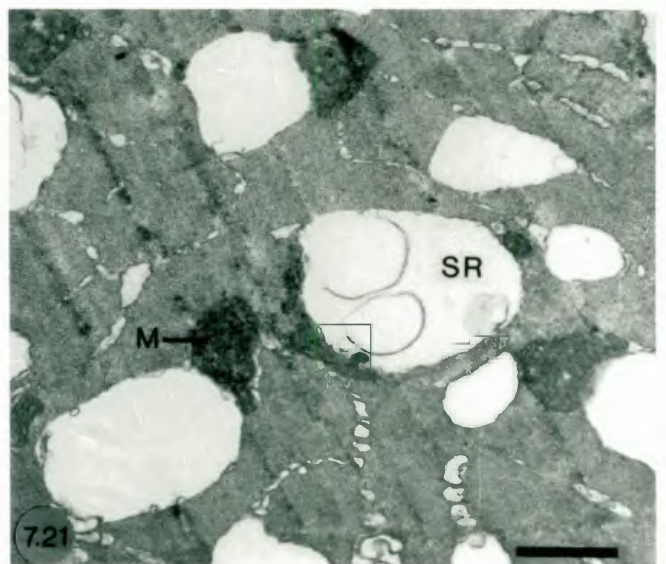
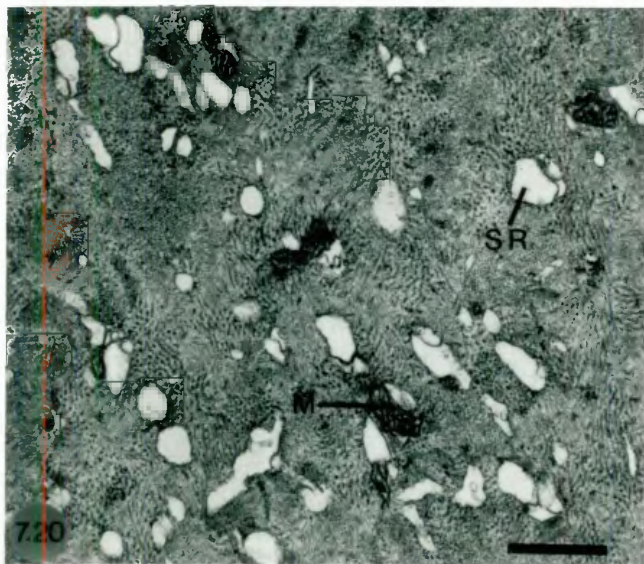
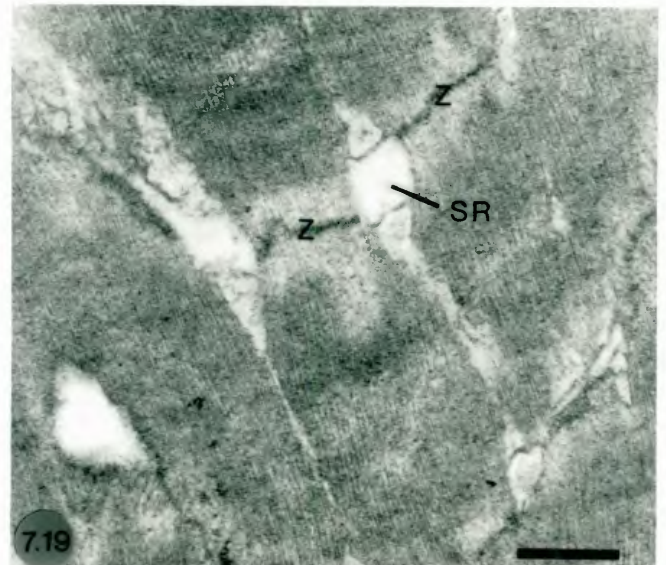
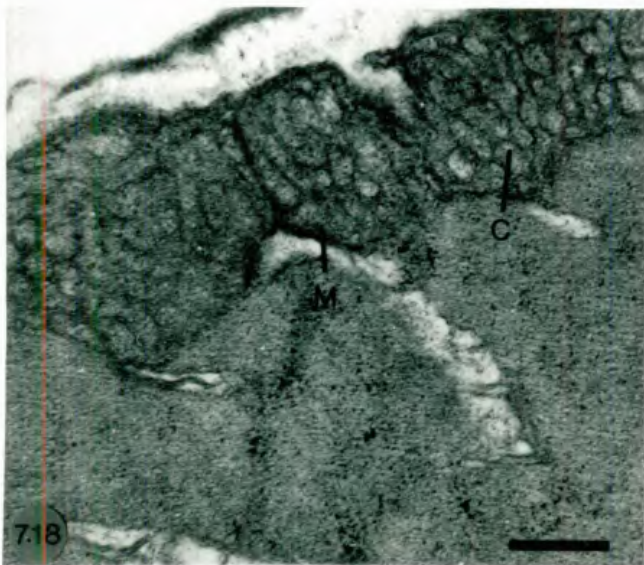
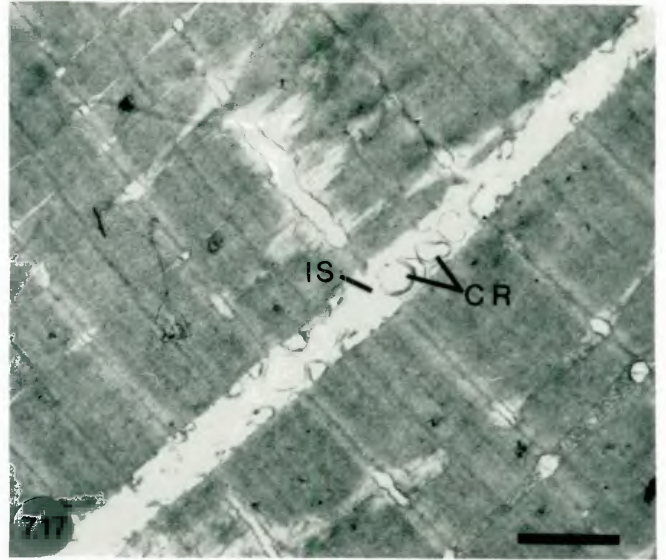
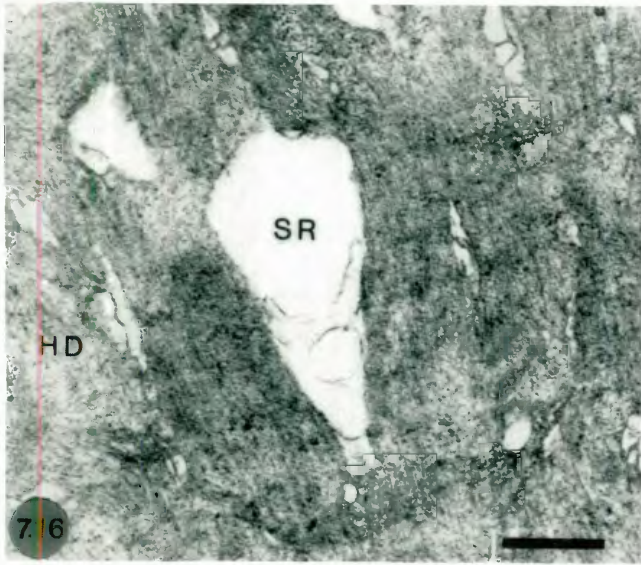


Figure 7.22: Sternohyoideus muscle from *Dascyllus aruanus*, image enhanced for image analysis use, showing normal muscle morphology (transverse section) SR - sarcoplasmic reticulum (Bar = 0.69 μm).

Figure 7.23: Binary image from image enhanced electron micrograph of control sternohyoideus muscle from *Dascyllus aruanus*, used for image analysis (transverse section).

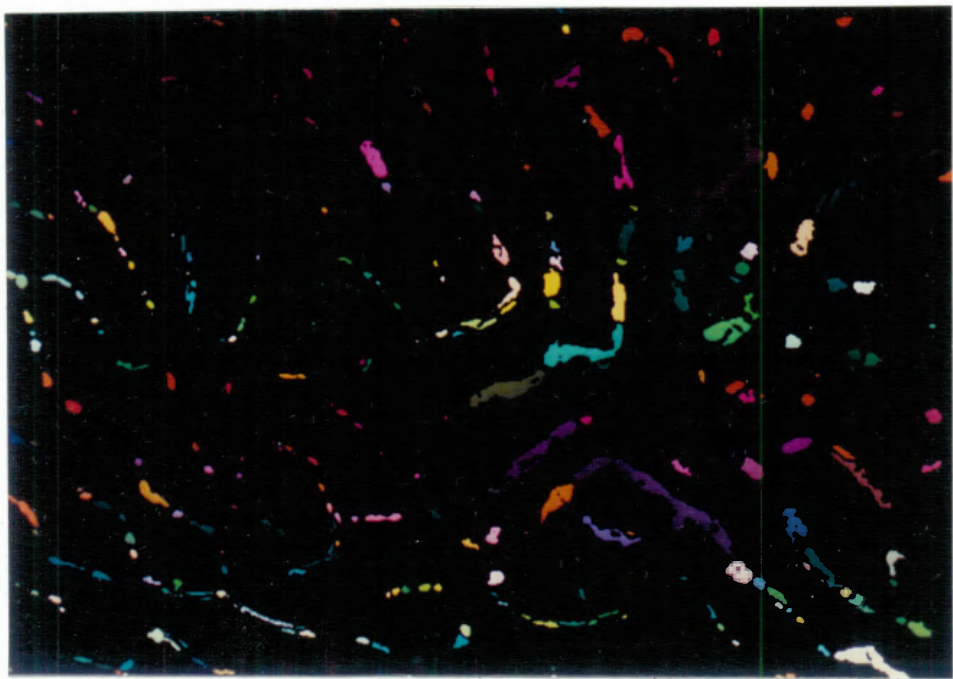
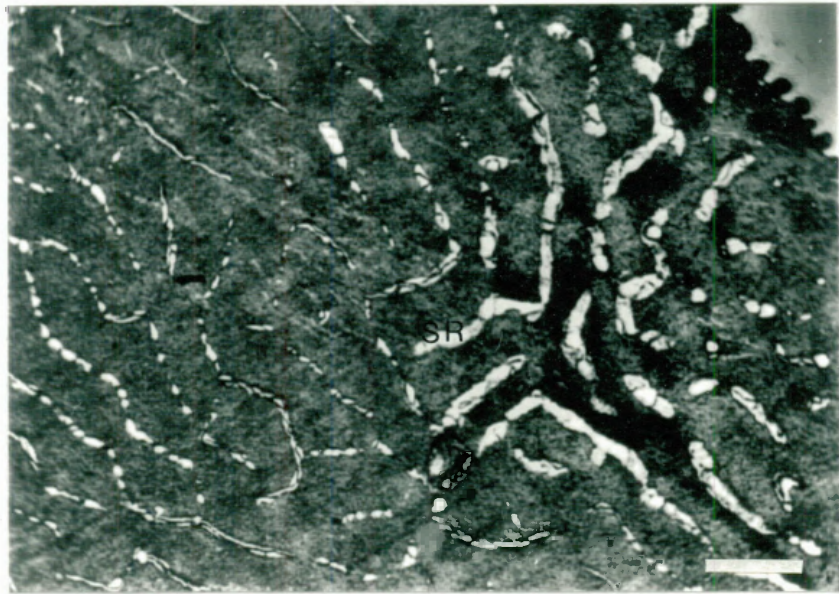


Figure 7.24: Sternohyoideus muscle from *Dascyllus aruanus* envenomated with *Aipysurus laevis* venom, image enhanced for image analysis use, showing swelling of the sarcoplasmic reticulum (transverse section) SR – sarcoplasmic reticulum (Bar = 0.69 μm).

Figure 7.25: Binary image from image enhanced electron micrograph of sternohyoideus muscle envenomated with *Aipysurus laevis* venom, used for image analysis (transverse section).

