Chapter 8

Venom Fractions

8.1 Introduction

Snake venoms are complex mixtures of proteins, as described in section 1.1, among which are toxins. Once such a heterogeneous solution is injected into an experimental subject a variety of physiological effects take place, either concomitantly or at different times. Any analysis of a given event provoked by the envenomation results from the summation of the effects of the different components. In order to assign precisely the origin of the effects, it is of the utmost importance, therefore, to proceed to fractionation of the whole venom, and to independently analyze the effects emanating from each fraction.

The present study is devoted to the examination of the effects of the fractional components of *Aipysurus laevis* venom on its prey species, examining ventilation rates, behavioural changes and muscle ultrastructural changes resulting from them.

8.2 Materials and Methods

Lyophilized *Aipysurus laevis* venom (50 mg) was dissolved in 2 ml 0.02 M ammonium acetate (pH 7.02). The venom solution was applied to a Bio-Rex-70 ion exchange column, equilibrated in the same buffer, and attached to a Waters HPLC system. The column was washed with the loading buffer until the absorbance at 280 nm

returned to baseline. At this point a linear gradient of 0.02 to 0.05 M ammonium acetate was applied over 300 minutes. Absorbances were obtained using a Waters 990 Photodiode Array Detector, which monitored column effluent. The ammonium concentration gradient was controlled with a Waters Automatic Gradient Controller (model 680), and an LKB Superrac maintained a flow rate of 1 ml / min, with 2 ml samples collected throughout. Venom fractions were pooled in glass containers coated with Coatasil (2293) water repellent silicone treatment (Ajax Chemical Pty. Ltd.) to prevent adhesion to glass. All the fractions were freeze dried (lyophilized) and stored according to procedures outlined in section 2.1.

Dascyllus aruanus (Fig. 2.8) were captured and maintained according to procedures discussed in chapter 2. The fish were injected as described in section 2.2, with the doses for the fractions determined from the proportion of the whole venom that they comprised. These proportions were calculated from comparisons of the areas that each fraction possessed, which were obtained from the chromatograph for the fractionation (Fig. 8.1). The whole venom dose to which the proportions were adjusted was 0.75 mg / kg. A proportional recombination of the venom fractions was constructed for testing with the fractions as a control to determine if the separation procedures affected fraction characteristics.

Ventilation studies and accompanying analysis of repeated measurements, for the venom fractions and fraction recombination envenomations, were carried out according to procedures outlined in chapter 5. Graphic representations of mean ventilation rates / minute over time, as well as comparisons of significance between the experimental groups were constructed.

Behavioural observations were carried out on the individuals for the fraction and fraction recombination envenomations in accordance with procedures discussed in section 6.2. An ethogram of behavioural responses was not constructed, with behavioural responses only summarized for comparative purposes.

Ultrastructural studies with sternohyoideus muscle were carried out according to procedures in section 2.7, for the fish envenomated with the seven different venom fractions. Comparisons between these results, previous ultrastructural results from fish envenomated with whole venom (Chapter 7) and control fish were conducted.

Samples were taken between 96 and 120 hours after envenomation, during a holding period employed after the initial 48 hour experiment.

8.3 Results

8.3.1 Chromatography

The *Aipysurus laevis* venom used in this study, which was collected from the Swain Reefs in January 1986 (Section 2.1), was shown by chromatography to possess six main fractions (fractions 1 and 3 through 7), as revealed in figure 8.1. The area designated as fraction 2 on the chromatogram (Fig. 8.1) was examined because of an interest in the slight rise in the profile present in that area. This same overall profile, as seen in figure 8.1, was obtained independently by Tremeau and Menez (pers. comm.) in 1987 at the Service de Biochimie, Centre d'Etudes Nucleares, Gif-sur Yvette, France, for *A. laevis* venom using a similar chromatography system. They found fractions 2, 5 and 6 (3, 6 and 7 in this study) to be toxic to mice, considering them neurotoxic.

Appendix H shows a three-dimensional plot for the chromatograph (Fig. 8.1) of the present study.

Maeda and Tamiya (1976) previously fractionated *A. laevis* venom, with CM–cellulose column chromatography, isolating four fractions. Of the four fractions one was not retained on the column, with the other three (toxins *Aipysurus laevis* a, b and c) proving to be neurotoxic. The three were determined as short-chained neurotoxins (Section 1.1.3) of 60 amino acid residues and were highly homologous; a and b differed by only two residues and a and c by one. The precise origin of this venom was not indicated.

Recently, Ducancel *et al.* (1988) reported, through the cloning and sequence analysis of cDNA's encoding neurotoxins of the venom of *A. laevis* from the Swain Reefs, similarities and differences with Maeda and Tamiya's (1976) findings.

After successful fractionation of the venom, the aim of the present study was to determine the biological effects of those fractions in hopes to gain better insight

into the role each fraction plays in the whole venom. From this information further fractionations and biological examinations of specific fractions could be carried out.

The biological studies employed for examining the properties of the fractions were as follows:

- Examination of ventilatory rate changes as the result of envenomation.
- Examination of behavioural changes as the result of envenomation.
- Examination of ultrastructural changes in muscle as the result of envenomation.

Table 8.1 shows the venom dosages used during the study for each fraction, based on the proportions of the whole venom that each fraction comprised. The dosages were adjusted to an equivalant whole venom dose of 0.75 mg / kg.

8.3.2 Ventilatory study with fractions

Figure 8.2 reveals the profiles of average ventilation rates for *D. aruanus* envenomated with seven fractions of *A. laevis* venom, a recombination of those fractions and a control group for the entire 48 hour (2880 minutes). Figure 8.3 shows the first 150 minutes on a larger scale, allowing for easier interpretation of the initial observations.

No mortalities occurred among the controls or fraction groups 1 through 5. Mortality was observed, however, for fraction 6 and the fraction recombination groups, both demonstrating similar profiles to that of the higher venom dose groups of *D. aruanus* (Figs. 5.3 and 5.4).

As with the ventilation study of chapter 5, these results can be divided into three divisions of ventilation change, as described in section 5.3.2 and seen in figure 5.13. The initial effects of division 1, exhibiting a high ventilation rate, are the result of subject manipulation, subject's lack of familiarization with the experimental conditions and observer's presence. The rates of all groups decreased with time (Fig. 8.3). The increase in ventilation rates marking the start of division 2 (possible primary effects of the whole venom) occurred from 15 to 120 minutes for fractions 1, 3, 5, 6, 7 and the recombination groups, with no significant ($P > 0.05$) ventilation increase after division 1 for the controls and fraction groups 2 and 4 (Figs. 8.2 and 8.3). The area

corresponding to division 2 of figure 5.13, possesses high ventilation rates as the result of the primary venom effects (Section 5.3.2). The most significant ($P < 0.01$) increase in ventilation rates belongs to fraction 6 and the fraction recombination groups; these groups demonstrated no significant difference between each other for the majority of the division. The next highest ventilation rate profiles for division 2 occurred with fraction groups 1, 3 and 5, with no significant difference ($P > 0.05$) between the groups, but significantly different ($P < 0.01$) from the controls for the majority of the division. Fraction groups 2 and 4 are significantly lower ($P < 0.01$) than the controls and demonstrated no significant difference ($P > 0.05$) between each other 95% of the experimental time. Division 3 of figure 5.13 represents the residual or long-term effects of the whole venom, as the result of the minor venom components. Ventilation rate profiles decreased during this latter stage, as the primary effects diminished. Ventilatory rates for fractions 1, 5, 6, 7 and the fraction recombination, however, were still significantly different ($P < 0.01$) from the controls, indicating secondary effects of the envenomations. Fraction 6 and the fraction recombination group became significantly lower than the control group, representing the profiles of dying fish. Fraction groups 2, 3 and 4 demonstrated an increase in similarity to the control during this division, all being non-significant ($P > 0.05$) at this time (Appendix J). Other similarities between fraction 6 group and the fraction recombination group, i.e. variability and patterns of variability changes throughout the experiment, can be ascertained from table 8.2. The differences in variability from other fracton groups can also be determined from table 8.2. This table of means and standard deviations was obtained from the data of ventilation rates for the eight groups.

Appendix I, containing probability values from the LSD calculations of ventilation rate comparisons, was used to construct the graphic profiles for the experimental group comparisons seen in Appendix J. These profiles were used to determine non-significances that existed between the groups. Table 8.3 was constructed from those probability comparisons and shows the percent of non-significance that existed between the groups for the entire experimental time.

The greatest similarities between the ventilation profiles of experimental groups occurred between fractions 2 and 4; they were similar 95% of the experimental time.

The least similarities were seen between 4 and 5, 2 and the recombination group and 4 and the recombination group (0% similarities), groups 2 and 7, and the control and the recombination group (1% similarities) and groups 4 and 7 and the control and group 6 (2% similarities).

All groups showed varying degrees of difference from the control group (significant 40% to 98% of the time), indicating that all had some effect on ventilation. Even fraction 2, which only possessed a slight rise in its profile (Fig. 8.1), demonstrated a difference. The recombination group and fraction 6 were significantly different ($P < 0.01$) from the control over 98% of the experimental time. This indicates that these groups had the greatest effects on ventilation, and were similar to each other. Fraction 3 appeared to have the highest affinity with the control group, but it demonstrated differences from the control 40% of the time.

8.3.3 Behavioural study with fractions

The observed behaviours for the seven venom fractions and the venom fraction recombination groups were not always as expected on the basis of the previous behavioural observations for the whole venom groups (Chapter 6).

The behavioural responses for this study can be categorized as follows:

1. Those responses that were similar to those observed during the whole venom study.

2. Those responses commonly observed during this study, but not commonly observed during the whole venom study.

3. Those responses observed during this study, but never observed during the whole venom study.

Due to lack of continuity between the experimental group's behavioural responses, it was not possible to construct an ethogram showing a hierarchy of responses, as was achieved for whole venom (Chapter 6). Table 8.4, however, is a summary of most of the behaviours observed in this study, along with the frequencies of their occurrences

for each experimental group. Ventilation rate changes were not included in this table, but are discussed in detail in section 8.3.2.

8.3.3.1 Fraction 1 (Behavioural Study)

Dascyllus aruanus, envenomated with venom fraction 1 (Table 8.4), lacked behaviours typical of those observed in the whole venom study of chapter 6. The significant increase in ventilation (Figs. 8.2 and 8.3, Section 8.3.2, Appendix J) and activity, indicates this fraction as affecting the fish. Those fish observed with swimming problems, leaning and eventually lying on their sides demonstrated better control of fins, eyes and ventilatory mechanism movements than previously observed. Fish were observed sitting on the bottom, with pectoral fins folded to their sides and engaged in minimal activity. This was never observed during the whole venom studies. During later periods of the study, bottom rubbing and the production of white fecal material was evident, both described in Appendix F.

All signs of envenomation progressively decreased, with 100% survival of the fish at completion of the experiment.

8.3.3.2 Fraction 2 (Behavioural Study)

D.aruanus envenomated with venom fraction 2 (Table 8.4) demonstrated no ventilation rate increase. The ventilation rates were significantly lower than in the controls for the first half of the experiment, and slightly higher, but not significantly different for the remainder (Figs. 8.2 and 8.3, Section 8.3.2, Appendix J). An increase in activity of the fish, with over 55% demonstrating periodic shaking, indicated that the fraction had minor effects on the individuals. Besides the yawning-like mouth movements seen in a few of the fish, no other ventilation related problems were observed. Minimal leaning and bottom rubbing (Appendix F) was observed, with 75% of the subjects producing white feces. White eye, as described in Appendix F, was observed in 41% of the fish during the earlier stages of the study; it lasted for approximately 20 hours before eventually disappearing. This sign was never observed during the whole venom studies.

All signs of envenomation progressively decreased, with 100% survival by the end of the 48 hour study. The fish appeared in good condition and were very active upon completion of the experiment, and during the subsequent holding period.

8.3.3.3 Fraction 3 (Behavioural Study)

D. aruanus envenomated with venom fraction 3 (Table 8.4) demonstrated an increase in ventilation rates that remained significantly higher than those of the control group for the first half of the experiment (Figs. 8.2 and 8.3, Section 8.3.2, Appendix J). This indicated that the venom fraction had an effect on the fish. No increase in activity was observed, however, with yawning-like mouth movements proving to be the only ventilatory problem present. Minor bottom rubbing was observed, with the majority of the fish producing white feces (Appendix F).

One sign observed here, but not in the whole venom studies, was a change in tail positioning with respect to the body. The tail was observed to be in an upwards or sidewards position, as described in Appendix F, and was not involved in swimming. This sign lasted for approximately 22 hours for 41% of the subjects.

All signs of envenomation progressively decreased, with 100% survival reported at the end of the experiment. Within 96 hours of envenomation conditions worsened, with swimming problems and color changes observed in a few of the individuals. After 120 hours, ventilation problems, not seen during earlier periods, were noted in a few of the fish.

8.3.3.4 Fraction 4 (Behavioural Study)

D. aruanus envenomated with venom fraction 4 (Table 8.4) had ventilation rates that were significantly below those of the control group for the first half of the experiment, and slightly higher, with only minimal significance for the remainder (Figs. 8.2 and 8.3, Section 8.3.2, Appendix J). No major increases in fish activity were observed, with only one individual demonstrating any change. Yawning-like mouth movements were observed in 50% of the fish during the earlier part of the experiment, and gave way to progressively worsened ventilatory problems later. Skip breathing (Appendix F) appeared in 92% of the subjects, which was rarely observed in the whole venom

studies and never to such an extent. A decrease in mouth usage, during ventilation, followed in 58% of the subjects, with some of the mouths eventually remaining in an open, extended position.

Loss of mouth involvement during ventilatory shutdown was compensated by (1) an increase in ventilatory fequency of the opercular shields and (2) an increase in stroke volume, as seen by the exaggerated movements of the opercular shields (Section 1.3). Only minor problems with opercular shield functioning were noticed.

Based on the fact that no mortalities occurred, even with the cessation of mouth movements, the opercular shield movements successfully maintained water flow across the gills, providing for the necessary gas exchange to maintain metabolism. All subjects appeared in good condition, despite the ventilatory problems. When the fish reached this point during the whole venom studies (Section 6.3.1), none survived.

Fish displaying signs of mouth problems temporarily suspended overt evidences of them while feeding, but after feeding reverted to the prior signs within 30 seconds. This was never observed during the whole venom study, because fish never ate once they had reached this stage of envenomation.

Minor bottom rubbing (Appendix F), leaning and production of white feces was observed, with two fish developing white eye (Appendix F) for approximately 20 hours at the beginning of the experiment.

All fish survived the experiment, with signs decreasing in time. Near normal behaviours were observed during the holding period, with minimal skip breathing, observed at 120 hours, being the only exception.

8.3.3.5 Fraction 5 (Behavioural Study)

D. aruanus envenomated with venom fraction 5 (Table 8.4) demonstrated irregular ventilation rates throughout the 48 hour study (Fig. 8.2), with alternating ventilatory increases and decreases. This profile of ventilatory rates was never observed during the whole venom studies or with the other venom fraction groups. No increase of fish activity was observed, and except for yawning-like mouth movements observed in three of the fish, no major ventilatory malfunctions were seen. Stutter breathing (Appendix F) was noticed in one of the fish at the beginning of the experiment, with

skip breathing (Appendix F) noticed in another at the end. White fecal material (Appendix F) was produced by 70% of the fish, with white eye (Appendix F) strongly developed in 100% of the fish. The white eye appeared to be related to ventilation rate changes, whereby an increase in white eye seemed to parallel an increase in ventilation rate and vice versa.

All fish survived the 48 hour experiment, with most of the signs decreasing with time. During the holding period, two fish developed swimming problems, with one of them completely succumbing to the fraction's effects by 120 hours.

8.3.3.6 Fraction 6 (Behavioural Study)

D. aruanus envenomated with venom fraction 6 (Table 8.4) had the highest ventilation rates of any experimental group (Figs. 8.2 and 8.3). The rates were significantly different from the controls for the majority of the experiment (Section 8.3.2, Appendix J). A few of the fish had relatively high ventilation rates for long periods of time followed by a rapid drop of ventilation and subsequent death. Activity increases were observed during early periods of the study, but no yawning-like mouth movements occurred. Stutter breathing, as described in Appendix F, was seen in 83% of the fish during earlier experimental periods, with minimal skip breathing (Appendix F) also present. Both preceeded and then accompanied ventilation rate increases, and subsequent mechanism deficiencies, eventually decreasing in occurrence in those fish that survived the envenomation.

Fish that succumbed to the venom effects, demonstrated loss of swimming co-ordination and fin control, leaned to one side, eventually lying on the bottom, as previously described for the whole venom study (Chapter 6). However, these fish never attempted to correct changes in body orientation, whereas fish injected with whole venom attempted to do so quite frequently.

More severe signs of envenomation included problems with the normal movement of the parts of the ventilatory mechanism (i.e. mouth, opercular shields, opercular fringe). Envenomation with this fraction caused a change in the order of ventilatory shutdown from that previously described for whole venom studies in section 6.3.1. The present sequence was:

1. Opercular shields ceased movement and remained in an opened position.

2. Opercular fringe ceased to move.

3. Mouth ceased movement and remained in a closed position.

Not only was the order of shutdown reversed in this study (except for the fringe), but the mouth remained closed after it ceased to function in those that died. This rarely occurred during the whole venom study, the mouths of dying fish remaining in an open, extended position.

Another sign occurring during advanced stages of envenomation, both for this fraction and for the whole venom, was a darkening of the body pigmentation.

Tail degeneration was observed during more advanced stages of envenomation for those fish that died. Based on the fact that it was (1) rarely seen during the whole venom studies, (2) occurred only in those fish that died from envenomation, (3) occurred relatively quickly and (4) no whitespot occurred in the experiment, it is felt that tail degeneration probably was a response to the envenomation by fraction 6, rather than to other causes.

During the study, minor bottom rubbing was observed in two of the fish, but white feces were not produced. White eye was observed in 90% of the fish, with a prominent swelling of the entire eye observed in two of the fish during later stges. All are described in Appendix F.

The seven surviving fish showed decreasing signs with time and appeared healthier by the end of the experiment. They were not as active as the control fish, however, during the subsequent holding period.

There was more variation in response to envenomation by fraction 6 than to fractions 1 to 5, as revealed by statistical analysis of the ventilation rates (Table 8.2).

8.3.3.7 Fraction 7 (Behavioural Study)

D. aruanus envenomated with venom fraction 7 (Table 8.4) had ventilation rates that were not significantly different from those of controls during the first half of the experiment, but which became significantly higher for the remainder (Figs. 8.2

and 8.3, Section 8.3.2, Appendix J). Activity increased in all of the fish, and they demonstrated a high degree of aggression towards each other and the observer. They were observed to randomly rush at other fish, shaking their own bodies and attempting to bite. Although minimal aggression was observed in previous studies with whole venom, it did not occur to this extent and never towards the observer. These activities increased when the observer approached the aquarium more closely.

Yawning-like mouth movements were seen in a few of the fish, but no severe ventilatory problems were noticed. Stutter breathing was observed in 41% of the fish, but led to no further ventilatory problems. White eye was observed in 41% of the fish, with minor bottom rubbing also occurring. All of the fish produced white feces at one time or another during the experiment. All of these signs are described in Appendix F.

All signs decreased toward the end of the experiment, with 100% survival occurring. One of the fish laid on its side during the holding period, but otherwise normal ventilatory rates and movements were maintained.

8.3.3.8 Fraction recombination (Behavioural Study)

D. aruanus envenomated with the recombination of the fractions (Table 8.4) possessed a similar ventilation rate profile to that of fraction group 6 (Figs. 8.2 and 8.3), and *D. aruanus* envenomated with the whole venom (Chapter 5). There was no significant difference between the recombination and fraction 6 groups in ventilation rate profiles during 67% of the experiment (Section 8.3.2, Appendix J), the largest percent of non-significance for the comparisons between experimental groups (Table 8.3).

The increase in fish activity and ventilation rates paralleled those of the whole venom study (Chapter 6), with ventilatory problems and subsequent shutdown following the same sequences discussed in section 6.3.1. However, the order of shutdown of the ventilatory mechanism was different from that of the fraction 6 group.

Problems with swimming and maintenance of proper body orientation, were also similar to those observed in the whole venom study.

Stutter breathing occurred in 90% of the fish, white feces in 66% and two of the fish demonstrated swollen eyes near the end of the study. All are described in

Appendix F.

All of the signs decreased in severity by the end of the experiment. However, the effects of the envenomation were still observable during the holding period, as seen by some problems with ventilation.

8.3.4 Ultrastructural study with fractions

As with the previous venom fraction studies (Sections 8.3.2 and 8.3.3), these results were difficult to evaluate, because they differed from the results from the whole venom studies of chapter 7.

The following sections present ultrastructural examination of sternohyoideus muscle from *Dascyllus aruanus* envenomated with each of the seven venom fractions of *A. laevis* venom.

The summaries and micrographs depict examples typical of the many samples examined, representing early stages of envenomation (Ownby and Colberg, 1988). Perusal of the samples sometimes led to discoveries of minor signs, for particular fractions. These are mentioned in the text where appropriate, but are not figured.

The results were compared to normal skeletal muscle of control marine fish (see sections 1.2 and 7.3). More detailed information on ultrastructural changes of muscle, from fish envenomated by elapid and hydrophiid venoms, can be seen in sections 7.3 and 7.4.

8.3.4.1 Fraction 1 (Ultrastructural Study)

The ultrastructural examination of muscle from fish injected with fraction 1 (Figs. 8.4 to 8.9) showed minor swelling of the sarcomeres and sarcoplasmic reticulum, possibly resulting from the salt water buffers used (Section 7.3). The mitochondria and nuclei appeared normal, with minor shrinkage of the nuclei from the plasma membrane evident, which according to Stringer *et al.* (1972) is a sign of muscle degeneration.

The absence of any other more major signs of muscle degeneration, as described in section 7.3, indicates that muscle necrosis was not occurring; the minor shrinkage of the nuclei from the plasma membrane was possibly the result of a component that

specifically affects the nuclei, with no other myotoxic effect.

8.3.4.2 Fraction 2 (Ultrastructural Study)

Fraction 2 (Figs 8.10 to 8.15) commonly caused swelling of the sarcomeres and sarcoplasmic reticulum throughout the muscle. Intramitochondrial edema appeared to be present, possibly at an early stage, with disruption of cristae evident. The nuclei were elongated and pyknotic, with considerable shrinkage of the nuclei from the plasma membrane.

These signs are indicators of muscle degeneration (Stringer *et al.*, 1972; Harris *et al.*, 1975; Tu, 1977; Yokote, 1982) and this fraction is therefore thought to be myotoxic.

8.3.4.3 Fraction 3 (Ultrastructural Study)

Ultrastructural preparations of muscle from fish injected with fraction 3 (Figs. 8.16 to 8.21) showed no swelling of the sarcomeres and sarcoplasmic reticulum, or only minor swelling, possibly resulting from the salt water buffers (Section 7.3). The majority of the mitochondria demonstrated intramitochondrial edema with no cristae present. Mitochondria were not common in the samples and were assumed to have lysed as a result of envenomation. The nuclei appeared to be in early stages of change, with irregular shapes and the beginnings of elongation evident. Some shrinkage of the nuclei from the plasma membrane was evident.

The changes observed in the mitochondria and nuclei suggest muscle degeneration (Stringer *et al.*, 1972; Tu, 1977). The absence of any significant swelling of the sarcomeres and sarcoplasmic reticulum, however, does not fit the patterns for the onset of muscle necrosis described in chapter 7. Possibly only components specifically affecting mitochondria and nuclei are present in this fraction.

8.3.4.4 Fraction 4 (Ultrastructural Study)

Venom fraction 4 (Figs. 8.22 to 8.27) produced the highest degree of swelling of the sarcomeres and sarcoplasmic reticulum of any fraction. Distinguishing the triads of

the sarcoplasmic reticulum was difficult throughout the samples, with intramitochondrial edema present in advanced stages, and cristae totally absent. Mitochondria were few and were assumed to have lysed as the result of the envenomation. A lot of cellular debris was present, especially in interfibrillar spaces (not shown here), and were thought to be the result of muscle breakdown (Yokote, 1982). Glycogen granules (black dots in muscle) were common, and thought to be the result of mitochondrial inhibition (Stringer *et al.*, 1971; Tu, 1977). The nuclei were in early stages of elongation, with darkened areas, possibly becoming pyknotic; shrinkage of the nuclei from the plasma membrane was obvious. Z lines were difficult to distinguish, with Z line streaming (Ghadially, 1975) possibly beginning.

These signs were all clear indicators of the occurrence of muscle necrosis (Stringer *et al.*, 1972; Ghadially, 1975; Harris *et al.*, 1975; Tu, 1977; Yokote, 1982), and suggests this venom fraction as being myotoxic.

8.3.4.5 Fraction 5 (Ultrastructural Study)

No major ultrastructural changes occurred in muscle from fish injected with fraction 5 (Figs. 8.28 to 8.33). Sarcomeres were normal, but the sarcoplasmic reticulum appeared to have shrunken. This shrinkage closely resembled that of muscle from control goldfish (Section 3.3), despite the use of the salt water buffers (Section 7.3). Mitochondria were common throughout all samples, and appeared normal, with cristae present. Nuclei also appeared normal, with no shrinkage from the plasma membrane.

8.3.4.6 Fraction 6 (Ultrastructural Study)

Venom fraction 6 (Figs. 8.34 to 8.39) caused minor swelling of the sarcomeres, but, as in the case of the fraction 5, most of the sarcoplasmic reticulum appeared to have shrunk, despite the use of salt water buffers (Section 7.3). This fraction did have some minor effects on the sarcoplasmic reticulum, however, as seen by swelling that was observed at times throughout the samples (not shown here). Mitochondria appeared normal in shape, but cristae were disrupted. The nuclei appeared normal in shape, but with darkened areas, possibly becoming pyknotic; no shrinkage from the plasma membrane was observed.

No major muscle necrosis can be inferred from these results. However, some minor effects such as a few examples of sarcoplasmic reticulum swelling, cristae disruption and darkened areas of the nuclei were evident. All of these are indicators of necrosis (Stringer *et al.*, 1972; Tu, 1977), but the other signs of muscle necrosis that usually preceed these (Section 7.3) were absent.

8.3.4.7 Fraction 7 (Ultrastructural Study)

Venom fraction 7 (Figs. 8.40 to 8.45) caused no swelling of the sarcomeres, but there was shrinkage of the sarcoplasmic reticulum, as previously described for fractions 5 and 6. Mitochondria appeared normal in shape, but with possible minor disruption of cristae. The nuclei showed a few darkened areas, possibly becoming pyknotic, and there was minor shrinkage of the nuclei from the plasma membrane.

Fraction 7, like fraction 6, probably contains a minor necrotic substance, which on its own produces only minor signs. Possibly when such fractions are combined, as in whole venom, their effects are summed, with more severe signs produced. Synergism may be involved.

The shrinkage of the sarcoplasmic reticulum occurred only in response to fractions 5, 6 and 7, but not for the other fractions or for the whole venom. Possibly the effect is masked or counteracted by other components in the whole venom.

8.4 Discussion

As long ago as the late 1800's, scientists were trying to isolate the components of the venoms of Australian snakes (Lewis, 1978). Since then, many snake venoms have been fractionated, i.e. *A. laevis* venom was fractionated by Maeda and Tamiya (1976), and biochemical information acquired. However, work on the behavioural effects of such components has been minimal, and the role of specific fractions in subduing or killing prey is unknown.

After examining the effects of the whole venom of *A. laevis* on its prey species in this thesis, comparable studies with the fractionated components were then pursued.

Being complex mixtures, with the components probably having synergistic toxic effects (Barme, 1968; Chang, 1979; Heatwole, 1987), evaluation of the actions of whole venom is complicated. Study of venom fractions, however, allows isolation of the responses to particular components.

There were similarities of fish responses to whole venom and to some of the fractions. Many differences, however, were also observed. Possible reasons for such differences are:

1. The more potent neurotoxins in whole venom may be killing the subjects before the less toxic components could express their effects.

2. Components responsible for specific responses might be cumulative, with more than one component responsible for a particular effect. Each on its own, as in fraction form, would elicit a less intense response.

3. Inhibition. A component could cause a response while in fraction form, that would not occur in the presence of other components in whole venom.

4. Synergism. Two components together might elicit a different kind of response from either operating alone.

No signs of envenomation were caused by whole venom other than those produced by one or more of the fractions. This would indicate that any synergistic effect of the venom is quantitative, i.e. responsible for increasing the potentcy of the venom, rather than qualitative (creating additional effects).

All of the fractions had some effect on ventilation, but the nature of the effects among the fractions differed.

Fraction 6, which is known to possess a potent neurotoxin (Maeda and Tamiya, 1976), induced the greatest change in ventilation rates. The response paralleled that to the high dose of the whole venom study, and to recombined venom fractions. There were also similar patterns in variability and changes in variability of ventilatory responses during the experiments involving fraction 6, whole venom and the recombined venom fractions. A difference between fraction 6 and the whole venom group was that the former elicited less intense responses than the latter. Possibly, responses

to whole venom were the result of a cumulative effect of a number of fractions, each by itself (e.g. fraction 6) having a less intense effect on ventilation.

Fraction groups 2 and 4 produced ventilatory rates significantly lower than for the control for the first part of the experiment, a phenomenon never observed otherwise. Components responsible for this response were not expressed in the whole venom, either due to the components being overshadowed by powerful neurotoxins or inhibited by other components.

The high ventilation rates that were observed with many of the fraction groups during the last part of the study (Division 3), suggest the presence of other venom components. Perhaps these components are slow in producing their effects and their action only becomes evident after the effects of the other components have run their course.

Behavioural responses to fractions often differed from those of the whole venom. Responses that were similar between the fraction and the whole venom studies, i.e. ventilatory movements and body orientation, had lower intensities in the venom fraction studies. Being possibly cumulative in effect, some of the components would prove less potent alone than in combination with others. Fractions 4 and 6 both affected the movements of the ventilatory apparatus. The fraction 4 group did not demonstrate the complete sequence of responses normally seen in response to whole venom and no mortality occurred. The fraction 6 group, even though suffering mortality, had a lower intensity of responses. Possibly with the combination of fractions 4 and 6, the cumulative effects would intensify, and responses more closely resemble those to whole venom.

Fractions responsible for responses such as white feces production, bottom rubbing and skip breathing, which were observed to a much lesser extent in the whole venom study, may have been overshadowed by the potent neurotoxic effects of the whole venom. In fractions with less neurotoxic effect, the components responsible for eliciting these responses could express their effects.

Responses never observed before during the whole venom studies, i.e. stutter breathing, white eye, the extreme aggressive behaviour observed in the fraction 7

study and the changes in tail positions in the fraction 3 study may have been eliminated through early death by neurotoxins, or were possibly masked by more severe responses elicited by other components of whole venom or the recombined venom fractions. This latter explanation could possibly be one reason why the reversal of ventilatory shutdown, as seen in the fraction 6 study, was never observed during the whole venom study. Another possibility is that if different fractions cause different kinds of responses or influence the rates of responses, omission of one component may result in some effect being slowed and therefore appearing later in the sequence than if that fraction were present.

According to Yokote (1982), any variation from normal muscle morphology (Section 1.2) is a clear sign of a pathological condition. Pathological change of muscle in fish envenomated with *A. laevis* whole venom was noted in chapter 7.

Ultrastructural examinations revealed that responses to envenomation by fractions (1) were lower in intensity and (2) lacked the complete sequence of necrotic change (Stringer *et al.*, 1972) than was true for whole venom.

The lower intensity of response to individual fractions, may reflect only part of the cumulative potency of the components combined in whole venom. The response to only one component would be slower and would not cause as much ultrastructural damage.

The lack of the complete sequence of muscle necrosis may be related to cumulative effects, with signs not being expressed due to the absence of necessary components. In whole venom all components are there to satisfy this sequence.

For those fractions causing necrosis all but fraction 4 exhibited an incomplete sequence of necrotic events. Fraction 4 showed lower intensities than expected on the basis of the whole venom study (Chapter 7). Cumulatively these fractions would probably increase the intensity of the response and complete the sequence of necrosis.

The muscle necrosis demonstrated in the study of fractions represent beginning portions of the early phase (first division) of Ownby and Colberg's (1988) necrosis phase divisions (Section 1.1.4). Using whole venom at a comparable venom dose and sampling time, the late phase (third division) was seen (Chapter 7); the muscle appeared as a homogeneous amorphous mass, an advanced stage of necrosis.

No muscle necrosis was observed for venom fraction groups 1 and 5, and only nuclear disruption seen in fraction group 7. Fraction group 6 demonstrated minor signs of necrosis, with disruption of the cristae of the mitochondria and a few swollen sarcoplasmic reticulum dispersed throughout the samples.

Venom fraction groups 2, 3 and 4 demonstrated the presence of myotoxic components, with varying signs of muscle necrosis evident. Fraction 4 had the greatest necrotic effect, obviously possessing a major myotoxic component responsible for more advanced and complete necrosis. Fraction group 3 showed the least effect, with only the nuclei and mitochondria being affected.

Ultrastructural effects of recombined fractions were unable to be studied, due to limitations of finances and time. Similarities, however, of ventilatory rates and behavioural responses between this group and the one subjected to high doses of whole venom (Chapter 7) suggest that advanced necrosis might have been present.

The following summarizes the results obtained from fractions of *A. laevis* venom:

- **Fraction 1** had an effect on ventilation rate, possibly neurotoxic in origin, but no deterioration of the ventilatory mechanism was seen. No ultrastructural changes in muscle were observed.

- **Fraction 2** demonstrated no ventilatory changes, but signs of muscle necrosis were present, indicating the presence of a myotoxic component.

- **Fraction 3** demonstrated ventilatory rate changes, possibly neurotoxic in origin, but no problems with the ventilatory mechanism. Some signs of muscle necrosis were present, indicating a minor myotoxic component.

- **Fraction 4** demonstrated no ventilatory changes, indicating lack of neurotoxic components, but a strong myotoxic component is suggested by the presence of major signs of muscle necrosis.

- **Fraction 5** demonstrated an extremely irregular profile of ventilatory rates, suggesting presence of neurotoxin, but no problems with the ventilatory mechanism were observed. No ultrastructural changes of muscle were evident.

- **Fraction 6** possessed a potent neurotoxin, causing high ventilation rates and death of some of the subjects. A minor myotoxic component (possibly a phospholipase) is indicated by the presence of some swelling of the sarcoplasmic reticulum and disruption of cristae.

- **Fraction 7** demonstrated minor effects on ventilation rates, but no problems with the ventilatory mechanism. Minor ultrastructural changes indicate the possible presence of a myotoxic component.

- **Recombined Fractions** gave similar behavioural and ventilatory results to those of whole venom and of fraction 6. Ultrastructural changes were not studied.

In this study a better understanding of the components of *A. laevis* venom, their relationships to each other and to the whole venom was obtained. Much more work, however, is required for a better understanding of the function of the venom components and their biological effects.

The following topics would be valuable extensions of the present study.

1. The effects of the recombined fractions on muscle ultrastructure.

2. LD50 studies for each fraction, allowing for the examination of a range of doses. This would emphasize some responses and perhaps exhibit others not previously observed.

3. Different combinations. By combining fractions in different combinations, some of the synergistic effects, inhibitions and cumulative effects hypothesized in this study could be tested.

4. Refractionation of certain fractions believed to contain important, multiple components, and examination of their biological activities would lead to a much more refined understanding of the role of venom in subduing and killing prey.

Figure 8.1: Chromatograph for seven venom fractions obtained from lyophilized *Aipysurus laevis* venom dissolved in 0.02 M ammonium acetate (pH 7.02) and applied to a Bio-Rex-70 ion exchange column, with a linear gradient of 0.02 to 0.5 M (pH 7.02) ammonium acetate.

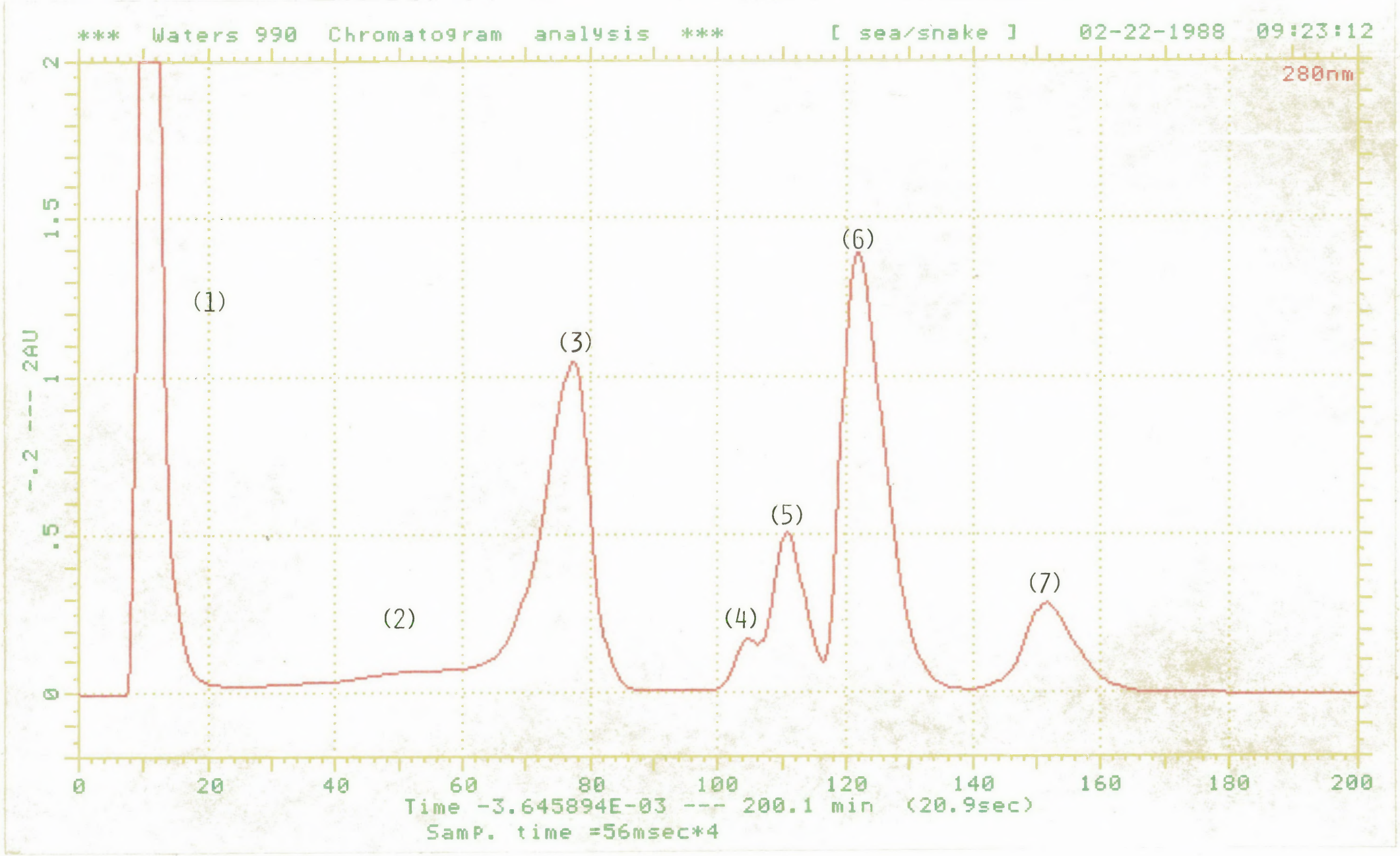
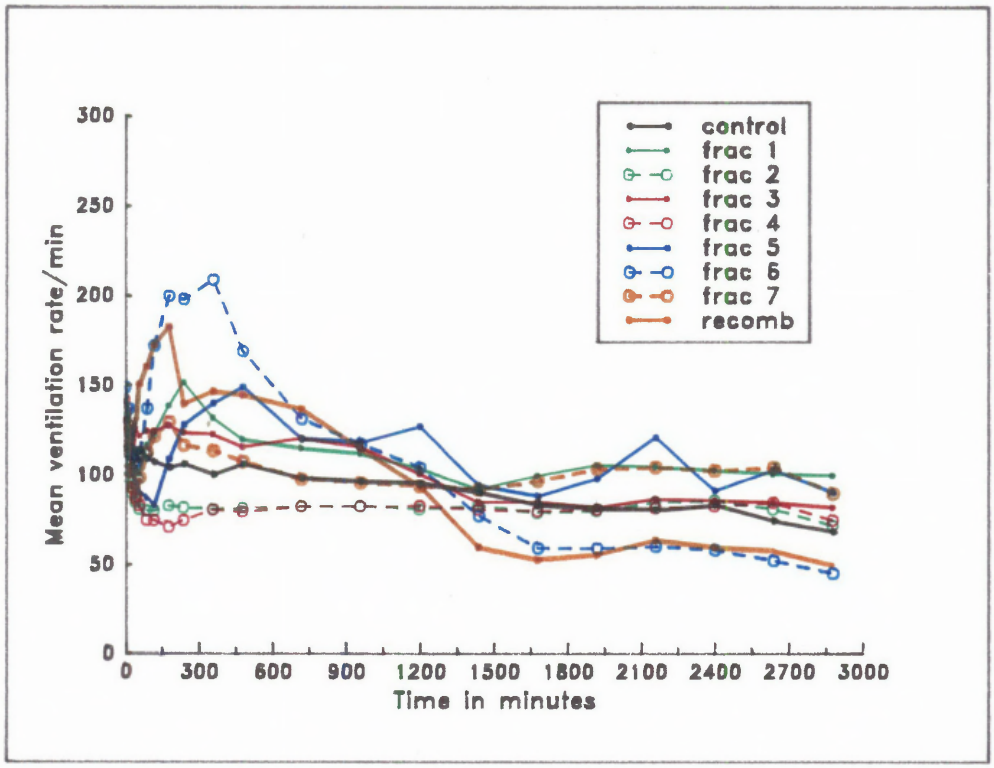

*** Waters 990 Chromatogram analysis ***
[sea/snake]
02-22-1988 09:23:12
280nm
2
1.5
1
.5
0
-.2 --- 2AU
(1)
(2)
(3)
(4)
(5)
(6)
(7)
0
20
40
60
80
100
120
140
160
180
200
Time -3.645894E-03 --- 200.1 min (20.9sec)
Samp. time =56msec*4

Table 8.1: Venom fraction dosages based on proportions of the whole venom that each comprise, as determined from the chromatogram for the fractionation. Doses are adjusted for an envenomation of 0.75mg/kg whole venom

Fraction No.	% of Total	Final Dose(mg/kg)
1	31.4	0.2355
2	4.4	0.0330
3	22.6	0.1695
4	1.7	0.0128
5	7.2	0.0540
6	26.0	0.1950
7	6.7	0.0503

Figure 8.2: Mean ventilation rates / minute after 2880 minutes of a control group of *Dascyllus aruanus* and of groups injected with venom fractions 1 through 7 and a fraction recombination.

Figure 8.3: Mean ventilation rates / minute after 150 minutes of a control group of *Dascyllus aruanus* and of groups injected with venom fractions 1 through 7 and a fraction recombination.


Mean ventilation rate/min
300
250
200
150
100
50
0
0
300
600
900
1200
1500
1800
2100
2400
2700
3000
Time in minutes
control
frac 1
frac 2
frac 3
frac 4
frac 5
frac 6
frac 7
recomb

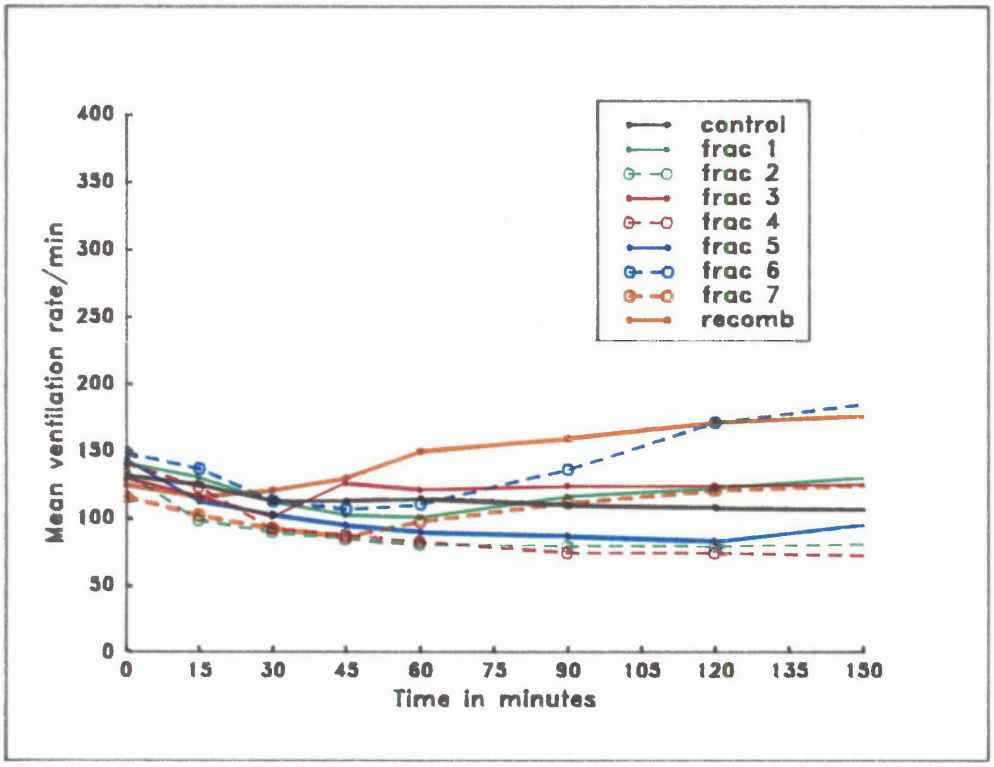

Mean ventilation rate/min
400
350
300
250
200
150
100
50
0
0
15
30
45
60
75
90
105
120
135
150
Time in minutes
control
frac 1
frac 2
frac 3
frac 4
frac 5
frac 6
frac 7
recomb

Table 8.2: Means ($\overline{X}$) and standard deviations (SD) for ventilation rate analysis for the venom fraction studies that examined the venom fractions (1–7), as well as the recombination of the fractions (n = 12).

Time in Min.	0	15	30	45	60	90	120	180	240	360	480	720	960	1200	1440	1680	1920	2160	2400	2640	2880
Fraction 1																					
$\overline{X}$	141	130	112	102	100	116	122	138	151	131	119	114	111	103	91	99	105	104	102	100	99
SD	9.7	12.4	12.6	9.5	6.9	14.6	10.6	12.2	19.0	20.6	9.0	6.3	6.8	4.9	6.2	5.3	7.1	6.1	4.7	3.9	6.0
Fraction 2																					
$\overline{X}$	134	98	89	84	80	79	79	82	81	80	81	82	82	80	82	78	79	84	85	80	71
SD	10.5	6.6	6.8	5.6	4.1	4.9	3.4	3.2	2.3	1.9	3.2	3.6	2.6	3.5	2.5	3.6	2.5	3.5	1.8	3.2	3.6
Fraction 3																					
$\overline{X}$	130	116	101	126	121	124	124	127	123	122	115	120	115	100	84	84	81	86	85	84	81
SD	12.8	8.2	5.0	7.5	3.9	9.3	2.8	6.7	11.4	10.0	9.3	13.9	12.4	6.2	3.0	3.4	2.7	4.7	3.4	4.7	3.7
Fraction 4																					
$\overline{X}$	141	123	92	87	82	74	74	70	74	80	79	82	82	82	80	79	80	81	82	84	74
SD	9.7	5.0	4.8	3.8	5.1	5.6	3.4	7.5	4.7	4.6	7.9	5.3	3.1	3.6	3.9	2.7	4.3	3.4	3.1	2.5	6.1
Fraction 5																					
$\overline{X}$	144	113	103	95	90	87	83	109	128	140	149	120	118	127	94	88	98	121	91	103	90
SD	12.5	9.7	5.8	6.4	5.6	4.4	3.8	10.8	16.9	18.8	20.3	8.1	13.0	16.4	8.7	8.3	9.2	8.4	7.6	8.3	5.1
Fraction 6																					
$\overline{X}$	149	137	113	107	111	137	172	200	198	209	169	131	117	104	77	59	59	60	58	52	45
SD	16.1	17.3	10.4	16.9	20.0	37.6	39.4	54.8	75.2	80.5	103.1	115.6	103.6	92.4	69.9	51.0	52.2	53.0	52.0	46.5	40.1
Fraction 7																					
$\overline{X}$	116	102	92	86	98	112	121	129	116	113	107	97	95	93	91	96	10.	104	102	104	89
SD	103	8.4	7.0	7.4	10.4	14.3	16.8	21.7	22.7	19.7	18.01	3.5	15.1	15.5	11.1	10.1	20.3	12.8	17.1	16.4	15.0
Recombination																					
$\overline{X}$	125	116	121	130	150	160	172	182	139	146	144	136	114	9.	59	52	55	63	59	57	49
SD	9.2	19.0	18.6	22.7	26.2	32.3	35.0	38.9	87.5	115.6	112.8	108.9	92.8	95.8	52.9	46.5	49.0	55.5	52.6	50.4	43.5

Table 8.3: Probability comparisons from LSD calculations showing % of time during the study that the experimental groups demonstrated no significant differences (P > 0.05) in ventilation rates. C – control, 1 – fraction 1, 2 – fraction 2, 3 – fraction 3, 4 – fraction 4, 5 – fraction 5, 6 – fraction 6, 7 – fraction 7, R – recombination of fractions.

	C	1	2	3	4	5	6	7
C	–	–	–	–	–	–	–	–
1	17	–	–	–	–	–	–	–
2	35	11	–	–	–	–	–	–
3	60	46	56	–	–	–	–	–
4	40	2	95	51	–	–	–	–
5	18	69	4	42	0	–	–	–
6	2	42	2	15	14	15	–	–
7	56	65	1	15	2	54	38	–
R	1	37	0	17	0	55	67	48

Table 8.4: Behavioural signs for fish envenomated with 7 venom fractions and one recombination of the fractions (R). The following refer to frequency of occurrence. – = no occurrence, 1 = light, (1-2 fish), 2 = med- light, (3-4 fish), 3 = medium, (5-6 fish), 4 = medium-heavy, (7-8 fish), 5 = heavy, (9-10 fish), 6 = very heavy (11-12 fish).

	Fractions							
Behavioural Signs	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**R**
Activity increase	3	4	–	1	–	6	6	6
Bottom rubbing	3	1	1	1	–	1	1	–
Yawning	–	2	3	3	2	–	2	–
Mouth problems	–	–	–	4	–	3	–	4
Problems opercular shield	–	–	–	1	–	3	–	3
Problems opercular fringe	–	–	–	–	–	3	–	3
Skip breathing	–	–	–	6	1	2	–	–
Stutter breathing	–	–	–	–	1	5	3	6
Swimming problems	1	–	–	–	–	3	–	1
Leaning	2	1	–	1	–	3	1	4
Lying on bottom	1	–	–	–	–	3	–	3
White feces	2	5	5	2	4	–	6	4
White eye	–	3	–	2	6	6	3	3
Swollen eye	–	–	–	–	–	1	–	1
Color change	–	–	–	–	–	3	–	–
Died	–	–	–	–	–	3	–	3